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PHYSIOLOGICAL REVIEWS

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CONTENTS

	PAGE
THE ADRENAL-GONAD RELATIONSHIP. *A. S. Parkes*	203
PHLORHIZIN GLUCOSURIA. *F. W. McKee and W. B. Hawkins*	255
THE PHYSIOLOGY OF THE PROSTATE GLAND. *Charles Huggins*	281
CEREBRAL CONCUSSION. *D. Denny-Brown*	296
THE APPRAISAL OF NUTRITIONAL STATUS (NUTRITURE) IN HUMANS. With Especial Reference to Vitamin Deficiency Diseases. *W. J. Dann and William J. Darby*	326
THE PROTEINS OF PLANTS. *Hubert Bradford Vickery*	347

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Tentative Contents of Volume 25, 1945

C. B. Huggins: Physiology of the Prostate

D. E. Gregg: Coronary Circulation

B. F. Miller: Biological Use of Detergents

Ernst and Berta Scharrer: Neurosecretion

E. B. Astwood: Control of Thyroid Function

H. E. Essex: Biological Reactions to Venoms

L. T. Fairhall: Inorganic Industrial Hazards

Harold F. Blum: Physiological Effects of Sunlight

Ralph Buchsbaum: Physiological Effect of Exposure to Cold (Frost Bite and Immersion Cold Effects)

Louis N. Katz: The Significance of the Electrocardiogram

E. J. Boell and J. S. Nicholas: Metabolism of the Embryo

F. C. Mann: Hibernation

George R. Cowgill: The Relative Value of Vegetable and Animal Fats in Nutrition

D. Denny-Brown: Concussion and Contusion in Relation to Loss of Consciousness and Amnesia

G. W. Beadle: Genesis and Metabolism in Neurospora

J. H. Welsh: Acetylcholine

Henry N. Harkins: Burns

H. B. Vickery: Protein Synthesis in Plants

Gerhard Schmidt and S. J. Thannhauser: Phospholipids and Lipidoses

C. Ladd Prosser: Invertebrate Neurophysiology

Mary Pickford: Control of the Secretion of Antidiuretic Hormone from the Pars Nervosa of the Pituitary Gland.

W. J. Dann and W. J. Darby: The Appraisal of Nutritional Status (Nutriture) in Humans

Bradley M. Patten: The Circulatory Transitions Precipitated by Birth

McKeen Cattell and Harry Gold: Digitalis Glycosides

F. W. McKee and W. B. Hawkins: Phlorhizin Glucosuria

Theodore Shedlovsky: Electrophoresis in Biology

W. C. Stadie: Intermediary Metabolism of Fatty Acids

Robert Chambers: Intercellular Cement and Permeability

Amedeo S. Marrazzi: Pharmacological Aspects of Synaptic Function

John W. Bean: Effects of High Oxygen Pressure

Isaac Schour: The Effects of Dietary Deficiencies upon the Teeth and Surrounding Structures

Otto Krayer: The Pharmacology of the Veratrum Alkaloids

David Nachmansohn: Cholinesterase

George Wald: The Chemistry of Visual Systems

J. F. Manery: Electrolytes of Tissues

H. S. Liddell: Experimental Neuroses

W. Feldberg: Acetylcholine as Transmitter in the Central Nervous System

D. B. Dill: Physiological Stresses in Aviation

R. A. Peters and H. M. Sinclair: Physiology of Vitamin B_1

John M. Buchanan and A. Baird Hastings: The Use of Isotopically Marked Carbon in the Study of Intermediary Metabolism

J. H. Burn: The Relation of Adrenalin to Acetylcholine in the Nervous System

A. S. Parkes: The Adrenal-Gonad Relationship

ERRATUM

Volume 25, 1945. Paper by J. W. Bean: Effects of Oxygen at Increased Pressure. Page 69, 2nd paragraph, line 14, which reads ". . . 137.5 + (3 x 360) + 1226.5 . . ." should read ". . . 137.5 + (3 x 360) + 9 = 1226.5 . . ."

PHYSIOLOGICAL REVIEWS

VOL. 25 APRIL, 1945 No. 2

THE ADRENAL-GONAD RELATIONSHIP

A. S. PARKES

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CONTENTS

I. Introduction 203
II. The effects of the gonads on the adrenals.
(a) Sex dimorphism in the adrenals 204
(b) Changes in the adrenals associated with the reproductive cycle 206
(c) Effects of gonadectomy 209
(d) Effects of gonadal hormones 211
III. The effects of the adrenals on the gonads.
(a) Effects of adrenalectomy 214
(b) Effects of adrenal extracts 216
IV. Adrenal-like activity of the gonads.
(a) Effects of sexual condition on survival after adrenalectomy 218
(b) Changes in water retention during the reproductive cycle 221
(c) Corticoid effects of gonadal hormones 222
V. Gonad-like activity of the adrenals.
(a) Gonad-like effects of the adrenals in experimental animals 224
(b) Adreno-genital syndrome 231
(c) Effects of cortical extracts and hormones on the accessory reproductive organs and secondary sexual characters 236
(d) Presence of gonadal hormones and analogous substances in the adrenals 239
(e) Excretion of androgens and oestrogens after gonadectomy and in adrenal dysfunction 240

I. INTRODUCTION. The existence of a functional relationship between the adrenal cortex[1] and the gonads was originally demonstrated by the clinical observation that disturbance of the adrenal glands might result in alteration of sexual functions or characters. In the last decade, however, many experiments on animals have shown that there is a close connection between the two organs, and the gradual working out of the details of the reciprocal interrelationship has been one of the most interesting phases of the expansion of endocrinological knowledge in recent years.

The evidence at the present time concerning the adrenal-gonad relationship is derived from the study, first, of the effects of the gonads on the adrenals and on the various physiological activities controlled by the adrenals, as shown by functional correlation, gonadectomy experiments and the injection of sex hormones, and secondly, of the effects of the adrenals on the gonads and on the accessory reproductive organs as shown by functional correlation, adrenalectomy experiments and the isolation of gonadal hormones and functionally analogous substances from adrenal cortical extracts.

Changes caused by the gonads in the adrenals and thence in those bodily

[1] Of the two components of the adrenal gland, the cortex only is referred to in this article, but for convenience the term adrenal gland is used throughout.

activities controlled by the adrenals must be clearly distinguished from direct corticoid activity on the part of the gonad hormones. Similarly, changes produced by the adrenals in the gonads and thence in the accessory organs must be clearly distinguished from direct effects produced on the accessory organs, either by the production in the cortex of androgens, oestrogens or progestogens, or by the gonad-hormone-like activity of the essential cortical hormones themselves.

In the review which follows, subdivision of subject is employed as far as possible to assist orderly presentation of the extremely heterogeneous mass of material. The subdivision cannot be complete, and some overlapping is unavoidable. Thus, discussion of the effects of corticosterone on the reproductive organs and of the presence of gonadal hormones in the adrenals is difficult to separate exactly from discussion of the effects on the reproductive organs of crude adrenal extracts, which may contain both of these substances. However, in the main, the aim has been to make each section self-contained. The literature discussed is that which appeared up to the end of 1941.

II. The effects of the gonads on the adrenals. (a) *Sex dimorphism in the adrenals.* Sex dimorphism of the adrenals is found in a number of species. It usually takes the form of a size difference, which may or may not be associated with obvious histological distinctions. Hill (193) makes the generalisation that the adrenals of the female at birth are heavier than those of the male in primates, carnivores and ungulates. Such a size dimorphism is, of course, accentuated by the fact that the female is usually of lesser body weight than the male. In rare instances the cortex in one sex or other possesses a special zone, either temporarily or permanently. There is as yet no conclusive evidence of sex dimorphism in function, except in some cases of neoplasia in humans, though it might be expected that the special zone which continues to develop in the female mouse, or which appears in pregnancy in certain species, performs some special function.

Fowl. Some difficulty is experienced in studying the adrenal glands of birds since clean dissection is difficult without undue manipulation, and the intermingling of cortical and medullary tissue complicates histological examination. Latimer (253) failed to find any difference in the weight of the adrenal glands in male and female fowl, but subsequently Sauer and Latimer (338) reported that the female fowl has approximately 30 per cent more cortical tissue, although the total weight of the gland is about the same.

Mouse. The best known case of sex dimorphism of the adrenals is to be found in the mouse. The whole gland is much larger in the female than in the male, as pointed out by Masui and Tamura (280) who ascribed the difference to the much greater development of the zona reticularis in the female. Moreover, the percentage of cortex is greater; according to Carlson, Gustafsson and Möller (62) the cortex comprises 82 per cent of the gland in the female, and only 67 per cent in the male. A more detailed study of the morphological difference was made by Howard-Miller (210) and by Deanesly (94) who reported that there was a much greater development of the inner cortical zone in the young

female mouse from the age of about five weeks onwards. Howard-Miller (210) introduced the term "X-zone" for this region, which consists of small darkly-staining cells, to distinguish it from the narrower and less sharply defined zona reticularis common to males and older females. According to Howard (205) mice of different strains vary greatly in the amount of X-zone tissue they develop, and, therefore, in the degree of sex dimorphism. Gersh and Grollman (154) failed to find any evidence that the X-zone performed a special function, and they regard it rather as a reserve source of cortical hormones.

Rat. Jackson (216) described the adrenal of the female rat as being larger than that of the male, and Hatai (185) came to a similar conclusion and remarked that the difference became greater with increasing body weight. Later, Hatai (186) recorded that the adrenals of the rat, up to a body weight of about 50 grams, are of about equal size in the two sexes. With increasing body weight sex dimorphism appears, and at 300 grams body weight the adrenals are almost twice as large in the female albino rat as in the male. Donaldson (106) gives curves which show that at 100 grams body weight the adrenal of the female albino rat is about one-third heavier than that of the male, while at 300 grams body weight the gland of the female is more than 50 per cent heavier. Donaldson (107) also studied the relative contribution of cortex and medulla to this sex dimorphism. King and Donaldson (221) found that in relation to body length the weight of the adrenal of the female rat exceeded that of the male by 90 per cent. The difference was only about half as great in captive gray Norway rats.

This gross sex dimorphism in the adrenals of the rat appears to have little qualitative histological basis. According to Howard (205) the "juvenile" cortex of the rat (see p. 206) shows no sex difference. In adults the zona reticularis is somewhat more distinct from the zona fasciculata in the female than in the male.

Guinea pig. Deanesly and Rowlands (100) found that the adrenal in the adult female guinea pig is larger than in the adult male guinea pig. According to Eaton (115) the adrenals are larger in the male and less variable. Zalesky (400) has recently stated that there is a histological sex difference in the adrenals of guinea pigs, the female having a somewhat wider zona fasciculata and a significantly narrower zona reticularis.

Rabbit. Roaf (327) reported that he was unable to find any clear histological sex dimorphism in the adrenals of the rabbit.

Cat. Latimer (254) found little difference in the weight of the adrenal glands of male and female cats when reckoned as a percentage of body weight.

Dog. Baker (11) noted that the adrenals were larger in dioestrous bitches than in mature males, but that the percentage weights were the reverse.

P. imates. Howard (203) made a detailed examination of the adrenals of Cebus and Ateleus monkeys but was unable to find clear histological sex dimorphism. According to Materna (281) and Freeman (140) the adrenal glands in women are slightly larger than those of men, a difference which is presumably accentuated when allowance is made for body weight. Parhon and Zugravu

(303) found the glands of the male to be much larger than those of the female in a series of 224 insane subjects.

(b) *Changes in the adrenals associated with the reproductive cycle. Pigeon.* According to Riddle (325) it is extremely probable that the adrenal glands enlarge considerably at the time of ovulation in the pigeon. The maximum size seems to be attained in the 44-hour interval between ovulation of the first and second eggs. Apart from gross hypertrophy the glands show no unusual features.

Mouse. The X-zone of the mouse develops about equally in the two sexes until the age of five weeks. In the male degeneration then takes place and the zone is replaced by fibrous reticular tissue which forms a capsule round the medulla. In the female this X-zone continues to grow until puberty when it occupies more than half the cortex in the unmated animal. It degenerates gradually in the unmated female and disappears before the end of the reproductive period, the histological changes being the same as in the young male. According to Howard-Miller (210) and Deanesly (94) there are no changes in the adrenal associated with the oestrous cycle of the unmated mouse, though Masui and Tamura (280) had previously reported that hypertrophy of the cortex occurred at oestrus. During pregnancy in the mouse, however, especially in the first pregnancy, there are marked changes in the adrenals. Tamura (374) reported a decrease in the gross size of the adrenal during pregnancy, although there is some hypertrophy of the zona glomerulosa and zona fasciculata, reaching a maximum at the 17 mm. stage. The most obvious change, however, occurs between the 7th and 12th days of the first pregnancy when the X-zone undergoes degeneration similar in degree and kind to that which occurs much more slowly in the unmated female.

Rat. Howard (205) made a detailed examination of the development of the adrenals of young rats and summarises her results as follows: "Rats of both sexes at three weeks of age show a differentiation of the inner third of the cortex, which differs from the adult zona reticularis, and resembles the mouse X-zone in some respects. This inner layer is here termed the juvenile cortex. The juvenile cortex, for the most part, is gradually transformed into the adult reticular zone, and has lost its distinctive character by forty days of age. Small groups of cells of the juvenile cortex may fail to undergo this differentiation, and persist as structurally isolated groups bearing some resemblance to the rat X-zone." Anderson and Kennedy (5) made a detailed study of the adrenal gland during the phases of the oestrous cycle in the unmated rat. They found that the relative and absolute weights of the gland are greater at oestrus than in dioestrus. The oestrus hypertrophy is purely cortical and is caused by enlargement of the cells of the zona fasciculata. Bourne and Zuckerman (25) obtained somewhat similar results in experiments which are described in more detail on p. 230.

During pregnancy, there seems to be but little change in the adrenal of the rat. Herring (189) found that the weight of the adrenal in the non-pregnant rat was 0.2 gram per kgm. body weight, which in pregnancy rose to 0.22 gram

per kgm., an insignificant change. Donaldson (108) was unable to detect any increase in weight during pregnancy and lactation in albino rats free from visible signs of disease. Donaldson (109) did not find any change in the relative volumes of cortex and medulla in the adrenal of the pregnant rat.

Guinea pig. Elliot and Tuckett (119) described the post-natal development of the adrenal of the guinea pig and drew attention to the relatively small size of the gland at birth and the great development of the cortex during adolescence. According to these authors the cortex, relative to medulla, is larger in the guinea pig than in any other mammal. Earlier, Guieyesse (170) had described enlargement of the cortex of the guinea pig during pregnancy—a result confirmed afterwards by Kolmer (229), Kolde (228) and Castaldi (67). Guieyesse described the change as being mainly in the cells of the middle of the cortex which increased in size and contained large vacuoles. Kolmer also reported that many mitoses occurred at the time of parturition. According to Verdozzi (387) the hypertrophy continues into lactation. According to Hewitt and Liere (191) the weight of the adrenal of the guinea pig increases relative to body weight in late pregnancy and again post-partum.

Ferret. It seems probable that the ferret, with a restricted breeding season and marked seasonal changes in the reproductive organs, would show corresponding changes in size in the adrenals, reflecting increase and decrease of hypophyseal activity, but no observations appear so far to have been recorded.

Rabbit. Early work on the development of the adrenal cortex of rabbits was carried out by Gottschau (163). Later, no hypertrophy of the cortex during pregnancy in the rabbit was found by Kolde (228) and Stilling (368). According to Randall and Graubard (321), however, there is considerable enlargement of the rabbit adrenal during the second half of pregnancy, together with an absolute increase in the amounts of phospholipid, free and ester cholesterol, neutral fat and total lipid. Only the neutral fat, however, increased in percentage amount.

Squirrel. Seasonal changes in the adrenal of the thirteen-lined ground squirrel have been described by Foster (135) who noted that the gland was small and poorly vascularised in anoestrus, the histological picture being one of inactivity. As the breeding season approaches, the glands, along with the gonads, increase in size by cellular proliferation and increased vascularity. The hypertrophy lasts throughout oestrus and pregnancy but involution starts shortly after parturition. Zalesky (399) also found that a significant increase in the size and weight of the adrenals, due to cortical hypertrophy, takes place in both male and female animals during the breeding season or when the gonads are artificially stimulated during anoestrus. Histologically he found that the hypertrophy consisted mainly of expansion and differentiation of the zona reticularis.

Mole. Kolmer (230) examined a number of pregnant and non-pregnant moles from February to April, at the beginning of the breeding season, and found adrenal enlargement at this time.

Opossum. Bourne (23) has described a remarkable hypertrophy of the

adrenal of the Australian opossum (*Trichosurus vulpecula*) during pregnancy. According to this author the cortex in this species cannot be divided into the three typical zones found in other mammals. Completely surrounding the medulla is a thin rim of cortex, divided into an outer fat-containing layer and an inner fat-free layer. These he terms "alpha" and "beta" zones respectively. In the male, both these layers remain thin. In the virgin female the beta zone begins to hypertrophy on one side of the gland and pushes into the medulla, and at maturity its inner cells on the hypertrophied side enlarge by cytoplasmic increase. During pregnancy there is tremendous hypertrophy of the whole of the zone.

Dog. Baker (12) found a significant hypertrophy of the adrenals during oestrus, but only in certain weight groups. Dioestrus was associated with decrease in the size of the gland. This author concluded that there was no hypertrophy of the adrenal during pregnancy or lactation and only slight hypertrophy during pseudo-pregnancy.

Sheep. Nahm and McKenzie (293) have described changes in the adrenal during the reproductive cycle in the ewe, the changes mainly consisting of an increase in the number of dark cells, and in the amount of lipoid during early oestrus and early and late pregnancy.

Primates. The relatively enormous size of the human adrenal at birth is well known; according to Hill (193) the fact that the gland at this stage is nearly as large as the kidney was known to Morgagni (1682–1781). The qualitative nature of the phenomenon was elucidated by the work of Starkel and Wegrzynowsky (362), Elliot and Armour (118), Kern (220) and Thomas (375). The gland at birth consists almost entirely of cortical tissue, the great size being due to the development in foetal life of a special inner cortical zone, to which the name foetal cortex is now applied. Immediately after birth this zone undergoes involution accompanied by extensive vascular degeneration and haemorrhage into the tissues. By the end of the second post-natal month the adrenal presents the essential structural features of the adult adrenal, the foetal cortex having given place to the zona reticularis. After the adult type of cortex is established there seem to be no definite changes associated with sex or reproductive condition.

In monkeys, the same pre- and post-natal changes seem to occur as in man. Hill (193) examined various species of macaques, adult and foetal, a foetal lemur and a foetal *Nycticebus*, and came to the conclusion that there was great hypertrophy of the foetal adrenal due to hypertrophy of the deeper cortical layers. This inner cortex, comparable to the foetal cortex of man, undergoes atrophy after birth. Hill notes that its successor, the zona reticularis, is also transitory in male primates. According to Howard (203) there are no changes in the adrenals of monkeys coinciding with puberty analogous to those seen in the mouse.

The adrenals of other mammals do not seem to show anything similar to the post-natal involution of the foetal cortex in primates, but the pre-pubertal degeneration of the "juvenile cortex" of the rat and of the X-zone of the male mouse may represent a similar process occurring at a later stage.

(c) *Effects of gonadectomy.* There have been many investigations of the effects of gonadectomy on the adrenals, but much of the work has been based on inadequate series of animals or upon unsuitable criteria. The literature up to 1933 is fully reviewed by Andersen and Kennedy (6) who concluded that much of the older work was of little value. Literature on the effects of ovariectomy on the adrenals was reviewed by Blumenfield (21). Only the more important of the contributions considered by these authors will be referred to in the present article.

Fowl. Soli (358) reported that the adrenals of capons were smaller than those of cocks of the same age, but Marrassini and Luciani (274) did not find any regular change.

Mouse. Altenburger (4) observed that castration increased the thickness of the adrenal cortex of the male mouse, an effect undoubtedly due to the stimulation of the X-zone which follows the operation (Masui and Tamura, 280; Howard-Miller, 210; Deanesly, 94). This effect is reversed by the grafting of testis tissue into the castrated male (Takewaki, 373). Prepubertal castration causes the adrenal of the male to approximate to that of the female, both in size and histological appearance, owing to the development of a darkly staining X-zone around the medulla. If castration is post-pubertal, the adrenal shows a similar reaction, but the X-zone is less well developed. The X-zone of the castrated mouse, like that of the female, gradually degenerates with increasing age. Gonadectomy has no effect on the growth or degeneration of the X-zone in the female.

Rat. Hatai (187) recorded extensive experiments on the effects of gonadectomy on the adrenals and concluded that the effects are opposite in the two sexes, there being an increase of size in males and a decrease in females. Andersen and Kennedy (6), however, have criticised Hatai's use of control material and maintain, from a recalculation of his figures, that the results were not so definite as suggested. Minowada (288) found a slight size increase in the adrenal of the castrated male rat, but was unable to detect histological change. Andersen and Kennedy (6) themselves concluded that the degree of the effect was dependent on the sex of the animal, the interval after operation and the age at operation. They found that nine weeks after castration of the immature male the adrenals were hypertrophied, contained more lipoids than the controls and histologically resembled the glands of the immature animal. Castration of the adult male rat produced no size changes and only transient histological ones. Ovariectomy of immature females resulted in the adrenals becoming similar in size to those of dioestrous controls. Ovariectomy of adult females caused a transient hypertrophy, so that the adrenals became similar to those of oestrous controls, followed by atrophy characterised by decrease of lipoid in the zona fasciculata and degenerative changes in the reticularis. A sex difference in response of the adrenals of the rat to gonadectomy was also recorded by Winter and Emery (394) who used sexually mature but not fully grown rats, gonadectomised for three weeks or more, and found hypertrophy of the adrenals in males and hypotrophy in females. Schilling and Laqueur (342) also noted a decrease in adrenal weight following ovariectomy of thyro-hyperplastic rats. The effects

of gonadectomy in rats has also been considered by Korenchevsky and his collaborators. Korenchevsky (231) and Korenchevsky and Dennison (236) found hypertrophy in the adrenals of the castrated male, amounting to about 25–40 per cent on a body weight basis. Hall and Korenchevsky (175) described this hypertrophy as being associated histologically with increase in width of the zona fasciculata and reticularis and in cell size and vacuolation. According to Hall (174) these histological changes, like the size changes, are absent in the adrenals of ovariectomised females.

Ellison and Burch (120) using adult females ovariectomised for twenty-five days, also noted atrophy of the adrenals. Lauson, Heller and Sevringhaus (255), however, did not find that a 20-day period of ovariectomy had any effect on adrenal weight in adult rats. Blumenfield (21) spayed rats shortly after weaning and killed them at three months old, and found that the adrenals were 8.3 per cent less than those of the controls, due to a 9.5 per cent decrease in the cortex. For rats killed at six months old the corresponding figures were 33.4 per cent and 35.8 per cent. These changes were associated histologically with cell shrinkage and cell destruction. Bourne and Zuckerman (25), however, state that cellular enlargement takes place in the zona fasciculata and zona reticularis in spayed rats, and that the adrenals, in the absence of the ovaries, undergo a 5-day cycle of changes similar to that seen in the normal female (Zuckerman, Bourne and Lewes, 410). Hypertrophy of the adrenal of the castrated male rat has also been noted by Chiodi (73) and Pinto (313). Howard (205) failed to find regular changes in the histology of the juvenile cortex of the rat following castration, but records one exceptional case of the development of a fairly well defined X-zone in a castrated rat. Waterman (390) found average adrenal weights of 49 $\pm$ 2.1 mgm. in female rats ovariectomised for one week, as compared with a control figure of 66 $\pm$ 2.6 mgm. Waterman adds the interesting observation that this decrease did not influence the survival time when the rats were subsequently adrenalectomised.

These records leave no doubt that the usual response of the adrenals of the rat to gonadectomy is hypertrophy in males and hypotrophy in females. These changes go far to abolish the sex dimorphism in adrenal size seen in normal rats.

Guinea pig. One of the more satisfactory of the early experiments on gonadectomy and the adrenals was carried out by Marrassini (273) on male guinea pigs. He found a hypertrophy due to vacuolation and increase of fasciculata cell size which was most marked at eight days after operation and was decreasing at 24 days. A somewhat similar result was obtained by Rigano-Irrera (326). Leinati (256) observed cortical hypertrophy in each one of a number of young guinea pigs killed from 10 to 80 days after operation. Moore (291), however, found that the adrenals were smaller in guinea pigs ovariectomised for long periods. A detailed investigation was carried out by Zalesky (400) who gonadectomized guinea pigs prepubertally and killed them approximately 18 months after operation. No appreciably permanent hypertrophy was found in either sex. The possibility of transient hypertrophy suggested by the work of earlier authors was not investigated. The sex differences in the adrenals (see p. 205)

persisted in the gonadectomised animals and are therefore either not produced or not maintained by gonadal activity. It may be said therefore that, so far as existing researches go, the guinea pig adrenal may show a transient hypertrophy after gonadectomy, but not a permanent one.

Rabbit. A number of indifferent researches have been carried out on the rabbit. Hypertrophy of the adrenals after gonadectomy was reported on very meagre evidence by Soli (358), Raineri (320) and Schenk (340), Friedl (144) for females, Kolde (228) and Livingston (263) for males, and Leinati (256) for males and females, and Zchwetadse (402) found some transient hypertrophy. Where histological studies were made the hypertrophy was said to be due to enlargement of the cortex, particularly of the zonae fasciculata and reticularis. On the other hand, Marrassini and Luciani (274) and Dick and Curtis (102) failed to find any effect. The former of these authors provided the most accurate and best controlled material.

Squirrel. Zalesky (399) found that castration of at least one month duration led to atrophy of the zona reticularis in the adrenal of the thirteen-lined ground squirrel.

Dog. Feodosyeff (129) reported hypertrophy of the adrenals after ovariectomy, but Pianese (312) in a controlled and apparently much more satisfactory experiment was unable to detect any change.

Sheep, cattle, pig and horse. A rather miscellaneous series of observations on spayed or castrated sheep and cattle were recorded by Marrassini and Luciani (274) but their material does not permit of definite conclusions. Giroud and Santa (156) reported that castration increases adrenal size in pigs, sheep, cows and horses, and also raises the ascorbic acid content.

(d) *Effects of gonadal hormones.* The observations recorded above show that there are undoubtedly changes in the adrenals associated with activity or lack of activity on the part of the gonads. If it be assumed that the connection is entirely endocrine it would be expected that the changes could be reproduced or abolished by the injection of gonadal hormones. This expectation has largely been realised, and, in addition, various effects of prolonged heavy dosage have been noted which indicate the possible effects of chronic gonadal hyperactivity. It is uncertain whether the changes are due to direct action of the gonadal hormones or to indirect action exerted through the pituitary body, which is known to undergo changes associated with the reproductive state of the animal and after gonadectomy. Such changes are commonly supposed to relate solely or mainly to the gonadotrophic function of the anterior pituitary body, but the supposition is difficult to prove. Experiments on gonadectomised-hypophysectomised animals are complicated by the fact that removal of the pituitary body causes severe hypotrophic changes in the adrenals, compromises their functional integrity and goes far to destroy their capacity to respond to stimuli other than the injection of adrenotrophin. Thus Deanesly's (95) observation that the X-zone does not develop in the pituitary-deficient dwarf mouse, either in the female or in the castrated male, undoubtedly means that the development of the X-zone is dependent on pituitary activity, but it does not show

whether the effect of the testes or injection of androgens in suppressing the development of the zone in the normal animal is brought about by preventing the cortex from responding to pituitary activity or whether the necessary pituitary activity is suppressed at source.

It is in the light of considerations such as these that the effects of injections of gonadal hormones on the adrenals must be assessed.

Mouse. Since the development of the X-zone is associated with the absence of the testis, it would be expected that the administration of testis extracts or androgens to the castrated male or the female would prevent the development of the zone or cause it to disappear. Martin (275) failed to find any effect of injecting "testicular hormone" for a few days, but observed that prolonged injections caused disappearance of the zone in castrated males and in females. A similar result was obtained by Poll (314) using an androgenic urine extract. Callow and Deanesly's (59) experiments with small doses of androsterone gave dubious results. Later, however, Deanesly and Parkes (98) obtained unequivocal positive results with 2 to 4 weeks injections into castrated immature mice, of the following substances and daily doses: androgenic urine extract (2.5 I.U.), testosterone (80 μg.), androstanediol (160 μg.), and *trans*-androstenediol (250 μg.). The last of these preparations was interesting in that it inhibited the development of the X-zone, but failed macroscopically to stimulate the accessory reproductive glands. The effectiveness of testosterone and testosterone propionate in suppressing the X-zone was also shown by Cramer and Horning (84), Starkey and Schmidt (363) and Tolenaar (382) who obtained the effect in castrated male mice, young mature females, immature males and females, adult females, and adult ovariectomised females. It is uncertain whether or not this effect of androgens is exerted directly on the adrenals or is due to modification of pituitary activity.

The effect of oestrogen on the mouse adrenal appears to be more complex. Martin (275) used a presumably impure preparation (oestrin) and found that over short periods it did not affect the X-zone. This result is in keeping with the observed fact that the development of the X-zone is independent of, and not apparently influenced by, the presence of the ovaries. Martin also found, however, that prolonged treatment of immature castrated males and normal and spayed females, led to degeneration of the zone. This result is perhaps analogous to the gradual disappearance of the zone seen in unmated normal females. Martin ascribed the effect to the depression of the gonadotrophic function of the pituitary body known to follow chronic oestrogenisation. His interpretation, however, presupposes that the gonadotrophic substance controls the development of the X-zone, whereas the contrary is indicated by Deanesly's (96) observation that the X-zone does not develop in the dwarf mouse in which, according to Smith and MacDowell (357) the gonadotrophic is the least deficient of the pituitary functions. By contrast, Martin also noted that prolonged injections of "oestrin" to normal immature males led to continued development of the X-zone. Burrows (42) also found that prolonged treatment of the normal male mouse with crystalline oestrogens led to the appearance of the

X-zone. This effect may have been due to the occurrence of a castrate-like condition following testicular atrophy caused by depression of the gonadotrophic function of the pituitary, but this interpretation assumes that chronic oestrogenisation does not affect the adrenocorticotrophic activity of the pituitary body, or whatever hypophyseal factor is responsible for X-zone development, or at least affects it less than the gonadotrophic activity. Cortical enlargement in mice oestrogenised for moderate periods has also been described by Deanesly (97). Results not dissimilar from those of Martin were obtained by Lacassagne and Raynaud (250) who found that oestrogenisation for short periods did not affect the X-zone, but that prolonged treatment produced changes in the adrenals ascribable to depression of hypophyseal activity. Degenerative changes in the adrenals of chronically oestrogenised mice have also been described by Cramer and Horning (85) and Danner (90).

The fact that the X-zone degenerates during the first pregnancy has led several workers to investigate the effects of corpus luteum hormone. Experiments were carried out by Martin with extracts of corpus luteum, which were found not to affect the condition of the X-zone. A similar result was also obtained for crystalline progesterone by Tolenaar (382) and Howard and Gengradom (211).

Rat. The sex dimorphism in size of the adrenals of the rat has been referred to on p. 205, the gland being larger in the female than in the male. We may now examine the extent to which this dimorphism is dependent on gonadal hormones. According to Korenchevsky, Dennison and Hall (239) testosterone does not have any effect on the size of the adrenals in normal male or female rats, but Hall (174) subsequently reported that "the effect of male hormones on females was to produce adrenals of similar size to those of normal uninjected males," thus causing the sex dimorphism to disappear. This conclusion agrees with that of McEwen, Selye and Collip (284) who found that injection of testosterone decreased the size of the adrenals in normal females by about one-third, thus reducing them to the same size as the glands in the male, which themselves were unaffected by the treatment. In keeping with this result it has been shown that in castrated males, the hypertrophy of the adrenals which follows removal of the testes (see p. 209) can be reduced or abolished by the administration of androgenic urine extract, androsterone, androstanediol, *trans*-dehydroandrosterone, testosterone, testosterone propionate, androstenedione and androstenediol (full references can be found in Hall and Korenchevsky 175). The histological appearance of the cortex is also restored to normal if moderate doses are used. The effects of androgenic substances in ovariectomised females are much less definite. According to Korenchevsky (234) injection of androgens often leads to some decrease in size, but this effect is less marked in the case of testosterone. As a general conclusion from these experiments it may be said that the sex dimorphism in the size of the adrenals of the rat is due largely to suppression of the male adrenal by testis activity, and that the effect can be reproduced in the gonadectomised male by appropriate treatment.

Comparatively little work has been done on the effect of oestrogens on the adrenals of rats. According to Selye, Collip and Thomson (348) enlargement

of the adrenals is only caused by oestrone when corpora lutea are present. Selye, Collip and Thomson (348) and Ellison and Burch (120) agree in stating that administration of oestrogens fails to cause hypertrophy of the adrenals in hypophysectomised rats, a result which indicates clearly the involvement of the pituitary body in the response seen in intact rats. Deanesly (97) noted that oestrogenisation of male rats leads, in the early stages, to cortical enlargement and, in the later stages, to atrophic changes consequent on the general disorganisation of pituitary function due to adenomatous changes. According to Korenchevsky and Dennison (235, 237) administration of large doses of oestrogens causes adrenal enlargement in normal male rats but not in castrates. It is likely that this effect is due to the coincident depression of testicular activity caused by the administration of oestrone, and the consequent appearance of castration changes in the adrenals. Lauson, Heller, and Sevringhaus (255) found that administration of oestrogens for 19 days did not affect the size of the adrenals in ovariectomised rats. Schilling and Laqueur (342) noted that injection of oestrone increased adrenal weight in thyro-hyperplastic female rats.

Squirrel. According to Zalesky, Wells, Overholser and Gomez (401) the administration of testosterone does not delay the atrophy of the adrenals which follows hypophysectomy in the ground squirrel.

III. The effects of the adrenal glands on the gonads. (a) *Effects of adrenalectomy.* Complete removal of all adrenal cortical tissue leads to death of the animal within a short time. Thus, pigeons (Gourfein, 164), fowl (Herrick and Torstveit, 188), and ducks (Parkes and Selye, 305, Bülbring, 33) survive only a few hours after the completion of the operation. In the rat, it was originally stated by many workers up to 1928 that a high proportion of animals survived indefinitely after adrenalectomy, but more recent work has confirmed the isolated earlier observations that adrenalectomy is almost always fatal in this species, the animals dying within a few weeks of complete removal of the adrenal cortical tissue. Thus, Pencharz, Olmsted and Giragossintz (308) found that all of 62 rats died within 2 to 18 days of the operation. Freed, Brownfield and Evans (139) and Kutz (248) made similar observations. Gaunt (147) emphasised the variation in different colonies of rats. In four different colonies, 95 per cent of the rats died within 3 weeks of double adrenalectomy, the average survival time being 7 days. In a fifth colony, 50 per cent lived for 30 days or more, the average survival time being 14 days for those that died. He observed no significant sex difference in survival time. Firor and Grollman (130) found that the mean survival time of the rat increased up to 9 days with increasing size of the animal. Schultzer (345) recorded a mean survival time in 77 rats of 5.7 days, with a range of 3–12 days. The older records of prolonged survival in rats after adrenalectomy appear to have been complicated by incomplete operations and the use of strains possessing much accessory tissue. Mice behave similarly (Firor and Grollman, 130). Most rabbits die within a short time of operation (11 days or less) but a few survive long periods probably owing to the presence of accessory tissue. In guinea pigs the complete operation is extremely difficult. For the cat, Elliot's (117) original observations, indicating a survival

period of 6 to 23 days, have been confirmed by many more recent workers, 7 to 11 days being the accepted average figure. Dogs survive about the same time, the average period shown by Rogoff and Stewart's (329) extensive observations being about 7 days.

In these circumstances observation of the effect of complete adrenal deficiency on the gonads and thence on the reproductive processes presents some difficulty, since observations must be made on completely adrenalectomised animals, in the short period before death, on animals suspected of having accessory tissue, or on animals kept alive on cortical extracts or corticosterone which may or may not affect the gonads.

Fowl. According to Herrick and Tortsveit (188) adrenalectomised fowl kept alive on cortical extract and sodium chloride showed testicular atrophy so markedly that in the course of a few weeks they had the appearance of capons.

Mouse and rat. Freed, Brownfield and Evans (139) adrenalectomised a series of male rats between 26 and 30 days old. At death a maximum of 6 days later, the testes were degenerating and smaller than those of the controls. In this type of experiment, however, it is difficult to distinguish specific effects on the gonads from changes due to the general failing condition of the animals. The observations of Lewis (258), Deanesly (94), Castillo (68) and Schiffer and Nice (341) on the length of the oestrous cycle and the breeding performance in adrenalectomised mice and rats were probably made on incompletely adrenalectomised animals, since the survival time was considerable. More convincing experiments were reported by Wyman (398) who adrenalectomised 41 mice and studied their oestrous history by the vaginal smear technique. Twenty-three died of acute adrenal deficiency and 16 of these had complete cessation of vaginal cornification in the month between operation and death. Examination of the ovaries showed that ovulation had also been suppressed. Eight rats died of chronic adrenal insufficiency and 6 of these had prolongation of the dioestrous period. Ten rats lived until autopsied 3 to 4½ months after operation, and 8 of these showed no serious disturbance of the cycle. Seven of this group were shown to have gross accessory cortical tissue. In another experiment, 28 adrenalectomised rats were mated, 15 became pregnant and 13 had normal litters. Eleven of these had gross accessory cortical tissue. Corey and Britton (83) found that the oestrous cycle was completely inhibited in 19 out of 22 adrenalectomised rats, but that it reappeared in adrenalectomised rats maintained on cortical extract. Kroc and Martin (244) record that of 18 adrenalectomised rats, 6 showed no return of the oestrous cycle after the removal of the second adrenal, while 8 showed one and 4 showed two oestrous type vaginal smears. Martin (276) obtained substantially similar results, and also found that adrenalectomy caused cytological changes in the pituitary gland and decreased the content of gonadotrophic hormone. The author attributed the reproductive derangements to the pituitary changes. This conclusion is in keeping with Dessau's (101) observation that the ovaries of doubly adrenalectomised rats respond normally to the administration of gonadotrophic extracts. However, Kutz, McKeown and Selye (249) showed that adrenalectomised rats maintained

in good health by the administration of sodium chloride experienced normal oestrous cycles, and Martin and Fazekas (277) found that adrenalectomised rats similarly maintained had a normal pituitary histology and gonadotrophin content as well as normal reproductive cycles.

The effect of adrenalectomy on pregnancy seems to follow the same principles. Britton and Kline (29) reported that pregnant rats which have been adrenalectomised fail to go through the normal processes of parturition, abortion is common, and lactation does not follow. Dessau (101) found that adrenalectomy of the rat in the first half of pregnancy terminates the pregnancy. Normal litters might, however, be produced if the operation were delayed until the second half of pregnancy. According to Tobin (381) normal gestation and parturition may proceed in the absence of the adrenals, the percentage of successes being proportional to the stage of pregnancy at which the operation is carried out. McKeown and Spurrell (285) found that adrenalectomy of the rat in the first 9 days of pregnancy led to non-implantation or to reabsorption; a salt diet usually prevented this result since most adrenalectomised pregnant rats maintained on salt delivered normal young, which, however, they were unable to rear owing to the failure of lactation.

It seems clear, therefore, that the effect of adrenalectomy on the ovary and on pregnancy, whether or not it is exerted through the pituitary body, is a result of loss of general condition rather than of the lack of a specific gonadergic substance produced by the adrenals. Most probably, as suggested by Long and Zuckerman (266), the changes in the accessory organs, involving considerable changes in their water metabolism, are dependent on a normal salt and water balance in the animal generally.

Cat and rabbit. Immediate adrenalectomy is said to inhibit the ovulation which would otherwise take place in cats following injection of gonadotrophic substances (Friedgood and Foster, 143) or after mating (Friedgood, 142), but the latter author inclines to the view that the effect is due to the general disturbance caused by the operation, since adrenalectomy of rabbits immediately after mating does not inhibit the ovulation which takes place some ten hours later. Adrenalectomy of the pregnant cat usually terminates pregnancy within 48 hours (Britton and Kline, 29).

(b) *Effects of adrenal extracts.* The work cited in the previous section makes it evident that the administration of cortical extracts allows the ovary of the adrenalectomised rat to continue its normal functions, but that the effect is a general somatic one and not due to special action of cortical hormones on the ovary. It remains now to examine the effects of adrenal extracts on the gonads and thence on the accessory organs of intact animals. The direct effects of such extracts, and particularly of desoxycorticosterone, on the accessory reproductive organs, are dealt with on p. 236 in dealing with gonad-like effects of the adrenals. The work to be considered in this section has largely been inspired by the observed facts of the adreno-genital syndrome in man and has dealt mainly with the effect on the gonads of (a) the typical life-maintaining extracts and substances of the adrenal cortex, and (b) other extracts made by methods designed to obtain different types of substance, if present.

Survival-prolonging extracts. The increasing availability of active (survival-prolonging) cortical extracts naturally led to experiments on the possibility of influencing the gonads of intact animals. Several apparently positive results were obtained, but these, as Grollman (169) points out, must have been due to chance, individual variation or non-specific constituents of the extracts. Certainly, more recent and carefully controlled experiments have failed to demonstrate any effect of cortical extracts on age of maturity, length of oestrous cycle or fertility.

Cleghorn (74) injected young mice daily for four weeks with a cortical extract made by the method of Swingle and Pfiffner. There was no significant change in the weight of the testis, the age of opening of the vagina or the length of the oestrous cycle as compared with controls. Schirrmeister (343) on the other hand, reported that repeated subcutaneous injections of adrenal cortex extracts in infantile mice produced enlargement of the reproductive tract and histological changes in its epithelium.

Corey and Britton (81, 82) claimed to have produced precocious maturation of the gonads in young rats by injection of Swingle and Pfiffner's extract. Changes were more marked in the ovaries than in the testis. Later, these authors (83) reported that administration of cortical extracts to normal rats might stimulate, depress or have no effect on the oestrous cycle.

Several other workers claim to have obtained positive results, including Fitzhugh (133), who found that cortical extract produced hypertrophy of the ovaries of young female rats; he did not, however, find that early maturity was produced in immature male rats. Hall, Chamberlin and Muller (176) reported that the administration of adrenal cortex extract to mature rats just before mating increased the incidence of pregnancy and the number of foetuses. The main action was on the female. The body of evidence, that cortical extracts as ordinarily prepared have no effect on the gonads, is, however, considerable. Connor (78) was unable to confirm Corey and Britton's early result. Gaunt and Parkins (152) treated young rats and fowl over long periods with daily doses of 5 to 20 dog units of survival-prolonging cortical extracts without influencing the reproductive system. Howard and Grollman (212) also concluded that a moderate excess of the hormone of the adrenal cortex has no appreciable influence on the size of the gonads, the oestrous cycle or on pregnancy. They also remarked, having in mind the masculinising tumours of the adrenal in man, that no signs of intersexuality occurred in the treated mice. Atwell (10) was unable to stimulate the ovaries of hypophysectomised rats by the injection of cortical extracts.

Pottenger and Simonsen (315) purified the benzene soluble fraction discarded in the process of preparing cortin by the method of Swingle and Pfiffner. They obtained a white amorphous powder which caused, they claim, increased testis weight and spermatogenesis when injected into male rats. Good results, which the authors consider may or may not have been due to the presence of "cortin," were also claimed in clinical trials on cases of cryptorchidism and arrested development of the testes and external genitalia.

Gonadotrophic extracts of the adrenal. There have been persistent reports

of experiments which suggest that some gonadotrophin-like substance is present in adrenal tissue. Thus, results suggestive of those obtained with gonadotrophic pituitary extract have been recorded by several workers who have used alkaline or similar essentially aqueous extracts of adrenal tissue. Casida and Hellbaum (66) found that pyridine extracts of the adrenals of mares and geldings caused ovarian stimulation in immature test rats. Allen and Bourne (2) made an aqueous extract of kangaroo adrenals and obtained luteinisation of the ovaries of immature rats. Similar results have been obtained by Hoffmann (198, 199) who described the active substance from the adrenals of cattle as having chemical properties very similar to those of pituitary gonadotrophins, and Kliatschko (223) who worked with extracts of pig, cattle and human adrenals, the last of these being much the most active. These authors emphasize that the gonadotrophic substance is distinct, biologically and chemically, from the recognised cortical hormones. Unfortunately, they have worked with rather minimal biological responses and whether or not the presence of small amounts of gonadotrophic substance in the adrenals is substantiated it may be taken as certain that the dramatic effects of aqueous pituitary extracts on the gonads are not simulated by similar adrenal extracts.

Miscellaneous experiments. Eaton, Insko, Thompson and Chidester (114) described an increase in weight of the testes of young chicks fed with desiccated adrenal cortex, while Chidester, Eaton and Thompson (72) conclude that similar treatment expedites sexual maturity in young female fowl. These conclusions receive no support from other knowledge as to the general nature of the active substances of the adrenal cortex. Klein (222) made a saline extract of adrenal cortex which he claimed stimulated the sexual organs of male rats and depressed those of female rats.

IV. Adrenal-like activity of the gonads. The first evidence that the gonads might, under certain conditions, show some adrenal-like activity was derived from the observation that, in dogs, the survival time after adrenalectomy was influenced by the sexual condition of the animal at the time of operation. More recently, the availability of large amounts of gonadal hormones in crystalline form has allowed the discovery that certain of these substances, notably progesterone, have corticosterone-like properties.

(a) *Effects of sexual condition on survival after adrenalectomy. Age and sex.* Sisson and March (353) found that female rats survived adrenalectomy slightly longer than males. The difference was slight, but was consistent at all age groups examined. They found that age, however, was a much more important factor than sex. Thus, at 20 days old, male rats survived an average of 5.0 days, females 6.4 days; at 70 days old, males survived 15.25 days and females 19.33 days. Gonadectomy experiments make it unlikely that the influence of age is connected with gonadal puberty changes. Kroc (243) also found that females survived slightly longer. He suggests that this could be associated with the larger size of the female adrenal, but it would seem equally reasonable to maintain that its larger size indicates a greater requirement on the part of the female and possibly, therefore, a shorter survival period after complete deprivation.

Gonadectomy. The effect of gonadectomy on survival after adrenalectomy has been examined by several workers. Carrying out castration simultaneously with adrenalectomy Carr (64) found a slightly longer survival period than after adrenalectomy alone, but it is doubtful if the slight difference had any significance. Mendez (286) carried out castration a few days before removing the adrenals and again obtained an insignificant increase in survival time. Since the size changes in the adrenals which follow gonadectomy in rats take some weeks to appear, experiments on previously castrated animals are of more interest than those in which the gonads and adrenals are removed simultaneously. Gonadectomy of male or female rats 6–20 weeks before adrenalectomy was found by Kroc (242, 243) to increase the survival period slightly (2–3 days) but insignificantly. Differences in weight loss were also small. In rabbits, Handovsky and Tamman (179) and Mira and Fontes (289) found that previous castration increased resistance to adrenalectomy. Kroc (243) notes that castrated male rats survive about as long as normal females, and suggests that this may be due to the fact that the adrenals hypertrophy in castrated males to a size approximating that seen in the normal female.

Anoestrus. A most interesting correlation between the condition of the gonads and the effects of adrenalectomy has been shown by Bülbring (34) who used drakes for the assay of cortical extracts. The drake is remarkable in showing a regular and very short survival period after the removal of the adrenals (Parkes and Selye, 305; Bülbring, 33), and Bülbring found that the amount of cortin required to keep the birds alive for a 16-hour period after operation varied greatly at different times of year. She was able to correlate the amount with the pronounced changes which take place in the size of the testes during the summer, the amount of cortin required being proportional to the size of the testes and decreasing by about five-sixths when the sudden drop in testis weight occurs during May and June. These results might be interpreted by supposing that testosterone is antagonistic to the action of corticosterone, but an alternative explanation is possible. The decrease in size of the drake testes after the breeding season is accompanied by atrophy of the seminiferous tubules, and by a change rather than a cessation of the endocrine activity. Male hormone effects disappear at the end of the breeding season, but, on the other hand, the plumage undergoes changes which are somewhat similar to those produced by oestrogens and which do not appear in the castrate, so that the small testes of the post-breeding season period must be supposed to elaborate substances different from those produced in the breeding season. It might be inferred, therefore, that the substance produced during the eclipse period can supplement corticosterone, rather than that the testosterone produced during the breeding season is antagonistic. Experiments on castrated drakes would, of course, settle this question.

A case of seasonal sensitivity to adrenalectomy in mammals was described by Britton (28) who showed that there is a marked difference in the survival of *Arctomys monax*, the North American marmot, according to the time of year at which double adrenalectomy is carried out. Hibernating marmots did not suffer

immediate ill-effects from adrenalectomy, but at the usual time of awakening in April such animals showed signs of severe adrenal insufficiency and soon died. The critical period clearly corresponds with the time of renewed activity in the gonads, but it is uncertain how far it is dependent thereon.

Oestrus. As recorded below the occurrence of oestrus at or about the time of operation in the dog, which ovulates and becomes pseudopregnant spontaneously, promotes survival after adrenalectomy. In the ferret, on the other hand, which does not ovulate spontaneously and remains in oestrus indefinitely in the absence of mating or other ovulation-producing stimulus, Gaunt and Hays (148) found that the occurrence of spontaneous or induced oestrus accelerated the appearance of symptoms of adrenal insufficiency.

Pregnancy and pseudopregnancy. Carr (64) found that the fact of a rat being pregnant did not increase its period of survival after adrenalectomy. Firor and Grollman (130), on the other hand, adrenalectomised ten pregnant rats and found an average survival time of 15 days, twice the usual period. These authors note that those rats which gave birth after the operation failed to come into lactation.

As long ago as 1912 Stewart (367) found that pregnant cats survived adrenalectomy longer than non-pregnant cats. Corey (79, 80) and Rogoff and Stewart (332), however, failed to confirm this finding, and Corey also found that lactation did not prolong survival time in cats. Collings (77), however, obtained very definite positive results in cats made pregnant or pseudopregnant following artificially induced ovulation. The average survival time following removal of the second adrenal was 23 days, nearly three times the average for non-pregnant animals. The discrepancies in these results on the cat are probably due to the fact that the cats were operated on at different stages of pregnancy. In the cat the corpora lutea begin to undergo involution about a month after the beginning of pseudopregnancy (Liche, 259) and considerably before the end of normal pregnancy. Since Corey used cats in various stages of pregnancy while Collings used them soon after mating, the mean prospective life of the corpora lutea at operation was probably very different in the two sets of experiments.

Rogoff and Stewart (330) found that pregnancy had a marked influence in prolonging the survival of dogs after adrenalectomy. Of 17 pregnant animals, 12 survived much longer than the average for males and non-pregnant females, the average being 22 days as compared with 7 days for the controls. These authors suggested, on histological grounds, that these results might be attributed to the presence of the corpus luteum during pregnancy. Later, Rogoff and Stewart (331) recorded that the occurrence of "heat" at or about the time of operation prolonged survival almost as well as pregnancy, survival periods of 36, 32, 19 and 64 days being recorded. They did not apparently recognise that heat in the unmated bitch ushers in a period of pseudopregnancy, which in this species lasts as long as true pregnancy and is associated with the same degree of development of the corpora lutea. This observation as to the effect of pseudopregnancy was confirmed by Pfiffner, Swingle and Vars (311) and Swingle, Parkins, Taylor and Morrell (370), and a similar state of affairs has been found

in another animal in which the pseudopregnant period is well developed—the ferret (Gaunt and Hays, 148, 149). It seems certain, therefore, that the foetuses are not involved in the prolonged survival of the pregnant animal after adrenalectomy. Swingle, Parkins, Taylor, Hays and Morrell (371) also showed that the natural occurrence or artificial induction of oestrus and pseudopregnancy in adrenalectomised bitches artificially maintained on cortical extract temporarily obviated the necessity for giving extract.

Stimulation of the ovaries with gonadotrophin. The researches outlined above, which concentrated attention on the corpus luteum as the corticomimetic tissue of the ovary prompted researches based on the mass production of corpora lutea by injection of gonadotrophic extracts. Thus, Emery and Schwabe (123) showed that the intensive luteinisation of the ovary of the immature rat by administration of pituitary or chorionic gonadotrophin prolonged the survival period for at least three weeks, and they showed that the effect was not due to production of oestrogens by the stimulated ovary, and was presumably, therefore, dependent on the corpora lutea. Administration of pituitary gonadotrophin to ovariectomised females or to males was without effect. Cavanaugh and Gaunt (70) got similar results by the implantation of rat pituitaries into young rats, but failed to do so with chorionic gonadotrophin. D'Amour and D'Amour (88) treated immature female rats first with mare serum extract, then with chorionic gonadotrophin, so that the ovaries increased up to 200 mgm. in weight. The animals were then adrenalectomised. The survival time was such that 50 per cent survived 20 days, as compared with an average of 5.2 days when the ovaries were removed at the time of adrenalectomy. Later, Pfeiffer and Hooker (310) showed that the treatment of female mice with mare serum gonadotrophin for 35 days before adrenalectomy greatly increased the survival rate. Longer periods of pre-treatment were not so effective, but this was probably due to decrease of the ovarian response as a result of the formation of antibodies to the gonadotrophin. Later, Emery and Gottsch (121) found that implantation of pituitaries of castrated rats, and to a lesser extent pituitary extracts, were also effective. Some doubt has been thrown on the explanation of the effect of pituitary extracts by the observation that they prolong survival not only in females, but also, though less readily, in entire and castrate male cats (Swingle, Parkins, Taylor, Hays and Morrell, 371, 372; Swingle, Parkins, Taylor and Morrell, 370). It should be remembered, however, that pituitary extracts contain active principles other than gonadotrophins, including at least one concerned with carbohydrate metabolism, and a survival-prolonging effect of such extracts, not mediated by the gonads, is therefore perhaps understandable. This suggestion is emphasized by the fact that mare serum extract, which so far as is at present known, contains no active principles other than gonadotrophins, is not effective in prolonging the survival of male and castrate mice (Pfeiffer and Hooker, 310).

(b) *Changes in water retention during the reproductive cycle.* Attention may here be drawn to the changes which occur in the water content of certain of the reproductive organs and secondary sexual characters, and to a lesser extent

in other organs, during the phases of reproductive activity. These changes are undoubtedly conditioned by the gonadal hormones, notably by oestrogens and they are reminiscent in a general way of those caused by corticoid substances, but are essentially dissimilar in that they depend largely on tissue specificity in response, or are paralleled in a small degree only by similar changes in other tissues of the body.

During the follicular phase of the normal cycle in the rhesus monkey there is an increase in the water content of the uterine endometrium (van Dyke, 386). In the pig-tailed macaque the development of the sexual skin into a large oedematous swelling during the follicular phase, and its sudden regression at the time of ovulation, causes changes in water metabolism sufficiently pronounced to be clearly reflected in the body weight and in the amounts of urine excreted (Krohn and Zuckerman, 245). The same retention of water presumably occurs in other monkeys, such as the baboon, in which there is a similar swelling of the sexual skin in the follicular phase, and to a lesser extent in rhesus monkeys where the sexual skin undergoes slight swelling, corrugation, etc. Analogous processes presumably take place in the comb and wattles of cocks when they enlarge in response to androgenic stimulation.

In women, a gain in body weight often precedes menstruation (Sweeney, 369; Okey and Stewart, 300). Less frequently, there is a generalised or local oedema preceding or accompanying menstruation (Thomas, 376; Atkinson and Ivy, 9). It is clear that such changes might be due to one or more of several factors, and their relation to the phenomena seen in lower primates is uncertain. However, Peterson and Milles (309) found that menstruation in normal women was accompanied by changes in the permeability of the skin capillaries, which may well be the mechanism by which the sexual skin oedema in monkeys is brought about. Moreover, Thorn, Nelson and Thorn (380), who studied the sodium chloride and water balance in normal women, found that there was a retention during the intermenstrual and pre-menstrual phases, and an increased excretion at the onset of menstruation.

(c) *Corticoid effects of gonadal hormones.* The lethal effects of adrenalectomy seem to depend primarily on disturbance of the salt and water metabolism, and Thorn, Engel and Eisenberg (378) have shown that the salt-retaining activity of various cortical substances is related in a general way to their effectiveness in promoting survival after adrenalectomy. The adrenals, however, also play an important rôle in the regulation of carbohydrate metabolism and work-capacity. These various activities of the adrenals are dissociable, as shown by the fact that the three best known of the many active substances isolated from the adrenal cortex have a different effectiveness in different directions. Thus, corticosterone and 11-dehydro-17-hydroxycorticosterone (substance E of Kendall) are very effective in influencing carbohydrate metabolism (Long, Katzin and Fry, 265; Ingle, 213) and work-capacity (Ingle, 214), but have little effect or even an adverse effect on the retention of sodium and water, and, therefore, on survival after adrenalectomy (Ingle and Thorn, 215); desoxycorticosterone has little effect on carbohydrate metabolism (Long, Katzin and Fry,

265) or in maintaining work-capacity (Ingle, 214) but is most active in retaining salt and water, and, therefore, in promoting survival (Thorn, Engel and Eisenberg, 379). The possible corticoid effects of the gonadal steroids must, therefore, be considered in the light of these dissociable functions of the cortex.

Oestrogens. Oestrogens are generally agreed to have no favourable effect on the survival of adrenalectomised animals. Martin (276), Swingle, Parkins, Taylor, Hays and Morrell (371), and Emery and Schwabe (123) obtained negative results with small doses of oestrogenic extract, as did Wells and Greene (393) with oestradiol, diethylstilboestrol, triphenyl ethylene and 4:4′dihydroxydiphenyl. According to Kroc (242), small doses of oestrone to non-ovariectomised females increased survival time (from 14.3 days to about 19 days) in one series of rats, but in another the survival time was inversely proportional to the dosage. Injection before as well as after adrenalectomy prolonged survival. Injection of oestrone to castrated and non-castrated adrenalectomised males decreased survival time. Injected females showed vaginal cornification, so that the presence of cortical hormones is not essential for this response.

According to Cavanaugh and Gaunt (70) and Pfeiffer and Hooker (310) oestrogenic preparations are not merely not beneficial but are actually toxic. Thorn and Engle (377) found that oestrone in substantial dosage had no effect on the survival of adrenalectomised dogs, though 17 mgm. of oestradiol caused retention of sodium and chloride in a female patient with Addison's disease and both oestrone and oestradiol had this effect in normal dogs. In an experiment of a rather different kind, Cramer and Horning (86) found that whereas administration of oestrogen after adrenalectomy decreased survival time, chronic oestrogenisation before the operation increased it.

Injection of oestrogens into ovariectomised monkeys leads to retention of water in the sexual skin swelling similar to that seen in the follicular phase of the normal cycle (Zuckerman, 407). This response is strictly specific in nature, since the administration of cortical extract, testosterone, or progesterone, does not maintain the sexual skin swelling after the administration of oestrogen is discontinued. According to Astwood (8) the injection of oestrogen to the spayed rat causes a transient rise in the water content of the uterus, which reaches a peak at 6 hours after injection and is back to normal in 12 hours. A similar result for the uterus was found by Zuckerman, Palmer and Bourne (411) who also showed that there were similar water-content changes in the skin, and reciprocal ones in muscle, heart, brain, pancreas and gut, the whole experiment being a conclusive demonstration of shift of water in the body caused by oestrogen administration.

Androgens. Testosterone is non-toxic and non-beneficial to adrenalectomised ferrets (Gaunt and Hays, 148). The same applies to androstenedione and dehydroandrosterone in rats (Wells and Greene, 393). Thorn and Engel (377) found that testosterone propionate had no beneficial effect in adrenalectomised dogs, though it caused retention of sodium in normal dogs. Androstenedione and androstenediol in the doses used (40 mgm.) were without effect even in normal dogs. Spurr and Kochakian (361) found that urinary androgenic extract,

androstenedione, testosterone acetate and testosterone propionate decreased the survival time of adrenalectomised rats, the effect being proportional to their androgenic activity.

Progesterone and allied substances. The evidence that the presence of functional corpora lutea ameliorates the effects of adrenalectomy, and the similarity in chemical structure between progesterone and the cortical substances, suggested that progesterone might give positive results in the ordinary tests for corticoid activity. Early investigators (Swingle, Parkins, Taylor, Hays and Morrell, 371; Cavanaugh and Gaunt, 70; Schacher, Browne and Selye, 339; D'Amour and Funk, 89; Steiger and Reichstein, 364) of this possibility obtained negative results, probably owing to the use of inadequate dosage. More recently a long series of reports of positive results makes it certain that the administration of large doses of progesterone promotes the survival of adrenalectomised animals. Two milligrams per day of progesterone maintained adrenalectomised ferrets in good health (Gaunt and Hays, 148, 150). It was less effective in adrenalectomised rats, 1 to 2 mgm. per day being required to extend life up to 20 days in half the animals (Gaunt, Nelson and Loomis, 151), and it did not modify their response to excess water. Greene, Wells and Ivy (168), however, found that a high proportion of adrenalectomised rats would survive on a daily dose of 4 mgm. progesterone. Pregnanediol and allopregnanediol have no corticomimetic activity (Wells and Greene, 393) in the Everse-de Fremery work-capacity test. Waterman, Danby, Gaarenstroom, Spanhoff and Uyldert (391) found that progesterone gave negative results in rats (4 mgm. daily) and in dogs (up to 12 mgm. daily). Ingle (214) also found that progesterone did not maintain the work-capacity of adrenalectomised rats. Thorn and Engel (377) report that 20 mgm. of progesterone to normal male dogs resulted in a decrease of sodium and chloride excretion about equal to that given by 0.8 mgm. desoxycorticosterone or 4 mgm. corticosterone, and according to Cantarow and Rakoff (61) progesterone has an effect similar to that of desoxycorticosterone in expediting the secretion of sodium and chloride into the peritoneum of normal dogs. In adrenalectomised rats, Schwabe and Emery (346) found that 1 mgm. daily of progesterone would maintain life, Bourne (24) that 0.5 mgm. daily of progesterone doubled the survival time, and Emery and Greco (122) that progesterone had the same order of activity in maintaining life as desoxycorticosterone acetate.

Work has also been carried out with compounds closely related to progesterone. Thus, Steiger and Reichstein (365) observed that 21-oxy-progesterone, in which a $CH_2 \cdot OH$ group is substituted for the terminal methyl group in progesterone, has definite corticosterone-like activity, about one-third as great as that of the substance itself.

V. Gonad-like activity of the adrenals. (a) *Gonad-like effects of the adrenals in experimental animals.* The clinical evidence that adrenal tissue can, under certain conditions, produce substances capable of inducing the development of rudimentary male organs and secondary sexual characters in the human female has prompted efforts to produce similar effects in experimental

animals. This work has mainly taken the form of attempts to demonstrate the secretion of androgens or oestrogens by the adrenals of male or female rats or mice. Owing to the absence of secondary sexual characters in ordinary laboratory animals, work has been concentrated on the accessory organs. This has made progress difficult in the male, since the prostate and seminal vesicles of rats and mice respond only to comparatively large doses of androgens.

Androgenic action in the male. A starting point for the work was provided by Price's observation (316) that whereas in the adult male rat castration leads to immediate degeneration of the prostate, especially of the epithelium, castration of the newborn rat is followed for some weeks by considerable growth and differentiation of the gland. In keeping with this observation Price (319) later showed that young ventral prostate tissue grafted into young castrated males undergoes development till the host reaches an age of about 40 days. Howard (204, 205) correlated the continued development of the prostate gland in the castrated newborn rat with cellular enlargement and other changes in the inner part of the adrenal cortex, and suggested that the adrenals of the young rat exerted an andromimetic function.

Burrill and Greene (37) in further analyses of this possibility, carried out experiments on young rats by *a*, castration; *b*, castration and adrenalectomy, and *c*, adrenalectomy. Adrenalectomy alone did not affect the early development of the prostate so that androgenic action by the adrenals does not seem to be essential in the presence of the testes, but removal of the adrenals in addition to the testes prevented the early differentiation seen after castration alone, and the authors concluded that andromimetic activity was exerted by the adrenals in the absence of the testes. Gersh and Grollman (155) carried out a similar experiment and arrived at contrary results. They were unable to find any difference between the prostate glands of castrated and castrated-adrenalectomised animals. Burrill and Greene (39) repeated their experiments on a larger scale and confirmed their previous results. Further, they claimed (41) to show that the adrenal androgen differs from the testicular one in not being destroyed by the liver. Howard (208, 209) added further evidence concerning this question. She found that adrenalectomy at three weeks old of castrated rats caused a 50 per cent decrease in weight of the prostate as well as degenerative histological changes. These changes in the prostate were not prevented when the rats were maintained at a normal growth rate by the administration of desoxycorticosterone acetate. She concluded that the adrenals secrete a physiologically appreciable amount of androgen at this stage of development. It may be noted that Burrill and Greene, and also Howard, maintained the adrenalectomised rats by sodium chloride therapy, while Gersh and Grollman administered adrenal extract, but it is unlikely that this difference can account for the discrepancy.

On the balance of the evidence it may be concluded provisionally that the adrenals of the immature rat are able to exert androgenic activity, though they do not apparently play an essential part in this respect if the testes are present. In the adult rat, however, it seems to be agreed that castration reveals no alternative source of androgenic substance. It has not been conclusively shown,

however, that this early differentiation of the rat prostate is dependent upon androgenic stimulation. Possibly, as Grollman (169) suggests, the rudimentary gland possesses some inherent growth potentiality.

The seminal vesicles of the rat behave differently from the prostate. According to Price (316) there is some slight enlargement after early castration, but both this author and Howard (207) agree that there is no differentiation. If the development of the prostate of the newborn castrated rat is really due to androgen of extra-gonadal origin, possibly the seminal vesicles have a higher threshold requirement.

If the adrenal glands of the normal young rat have androgenic activity it might be expected that glands caused to hypertrophy by the administration of hypophyseal adrenotrophic hormone would show the same activity more strongly. Actually, accounts of work on these lines preceded the papers by Burrill and Greene, and Gersh and Grollman. Davidson and Moon (93) first reported that the administration of adrenotrophic extracts to young castrated rats caused development of the prostate as seen by size increase and histological stimulation, and to a lesser extent of the seminal vesicles. The adrenals were much hypertrophied, and the authors attributed the changes in the accessory reproductive organs to the enhanced androgenic activity of the hypertrophied adrenals. In a later paper Davidson (92) showed that the adrenotrophic extract had no effect on the prostate if the animal was adrenalectomised but that the response was not abolished by hypophysectomy. These experiments, confirmed by Nelson (295), correlate well with the later ones of Burrill and Greene.

Less work has been done on mice, but according to Howard (204) there is a marked difference between the rat and mouse in the response to adult castration. In the rat, as mentioned above, degeneration of the prostatic and vesicular epithelium follows very rapidly after adult castration. In the mouse, on the other hand, although there is a considerable size decrease in both glands much of the epithelium of both glands retains its high columnar character and a fairly normal general character for as long as forty days after adult castration. The author ascribes this difference to the presence in the castrated mouse of the X-zone which is assumed to have greater andromimetic power than ordinary cortical tissue in the adult, and which mimics the testis and delays the post-castration changes in the accessory organs. In a more detailed paper, Howard (207) came to the following conclusions: "The reactions of the seminal vesicles of mice to castration vary according to the age at operation, and are related to the histological condition of the adrenal cortex. In mice castrated at 5 days of age, when the seminal vesicles are relatively undifferentiated, these structures undergo a marked degree of differentiation which includes the formation of columnar epithelium. This reaction is preceded by hypertrophy of the adrenal X-zone. In mice castrated at 21 days of age, when the columnar vesicle epithelium has differentiated, the X-zone hypertrophies and the condition of the vesicles undergoes little alteration for a considerable period, which may extend for as long as 100 days. In mice castrated after sexual maturity, when the primary X-zone has disappeared, the vesicles undergo a degeneration which remains incomplete

during the period in which the secondary X-zone differentiates in the adrenal cortex. One hundred days after castration the secondary X-zone has frequently disappeared from the cortex, and in these cases the vesicles are in a state of marked and uniform degeneration." Howard (206) later showed that adrenalectomy of young castrated mice prevented the differentiation of the vesicular epithelium which is not abolished by castration. A most interesting experiment bearing on this subject has been reported by Spiegel (360). He castrated three male guinea pigs a few days after birth. Several years later the rudimentary penis started to grow and two ultimately effected copulation, with the formation of vaginal plugs. At autopsy the reproductive tract and accessory glands were found to be similar to those of the normal adult male, and in each case there was a large adenoma of the adrenal cortex. This experiment seems to afford a clear case of androgen secretion in physiologically effective amounts by the hyperplastic adrenal.

Gynaecogenic action in the male. There is as yet little evidence suggestive of oestrogen secretion by the adrenal of the normal or castrated male mouse or rat. Oestrogen-induced changes in the male reproductive tract proper require large dosage and the males of these particular species are poorly equipped with female rudiments.

Androgenic action in the female. In considering the source of any androgens demonstrated to be present in the female, as for instance by Price's (317, 319) observations that male prostate tissue grafted into normal adult female rats remains in a functional state for several months, it must be remembered that the ovary has been shown to be capable of producing androgens. Thus, Lipschütz (260), Hill (192) and Deanesly (95) reported that under certain conditions ovarian grafts caused development of the accessory glands of castrated males, and Parkes (304) found slight androgenic activity in crude extracts of ovary. In spite of this complication there is now good experimental evidence that the adrenal of the female may show androgenic activity. The female rat is especially suitable for investigation of the endogenous production of androgens because it possesses a well marked male rudiment, a female prostate. These rudiments which were first described by Marx (278, 279) respond to androgenic stimulation in the same way as the male gland and are capable of development to a condition in which they are histologically identical with the ventral lobe in the male (Korenchevsky, 232; Korenchevsky and Dennison, 238; Korenchevsky, 233). The gland is not affected by oestrogens. Korenchevsky's observations were confirmed by Hamilton and Wolfe (178), Deanesly and Parkes (99) and Witschi, Mahoney and Riley (395).

Price (318) studied the development of this prostate gland in the female and found that, as in young castrated males, it undergoes growth and differentiation for a period of about 40 days, when regression begins. She pointed out that this period is similar to that during which the juvenile cortex of the rat adrenal retains its distinctive character in both males and females, and suggested that the early development of the female prostate is due to androgenic activity on the part of the juvenile cortex. Burrill and Greene (38) transplanted im-

mature male prostatic tissue into normal and ovariectomised immature females and concluded that the female prostatic tissue has a lower threshold requirement of androgenic stimulation than the male prostate, and that there is an extra-ovarian source of androgen in the female. Later the same authors (40) showed that neither ovariectomy nor adrenalectomy alone stopped the initial development of the female prostate, but that removal of both organs simultaneously did so. They concluded that both the ovary and the adrenal of the immature female rat are capable of producing physiologically demonstrable amounts of androgen.

Somewhat analogous observations have been made on the female preputial glands, which are only slightly affected by gonadectomy or injection of oestrogens, but which hypertrophy readily on the injection of androgens (Korenchevsky, 234). Noble and Collip (297) found that hypophysectomy caused atrophy and injection of pituitary extracts hypertrophy of the glands. The latter effect was almost abolished by the removal of adrenals and ovaries, and appeared to be mediated through the adrenals or ovaries, or both, according to the type of extract. It is likely, therefore, that under the influence of stimulation by pituitary extracts both adrenals and ovaries can produce enough androgen to cause growth of the preputial glands.

In this connection attention may be called to a highly suggestive series of experiments which are not usually considered in relation to the adrenal-gonad relationship. It is well known that under certain circumstances masculine stigmata, mainly hypertrophy of the clitoris and appearance of the preputial horns, appear in female guinea pigs. Steinach and Kun (366) produced this result in association with extensive luteinisation of the ovary following x-irradiation or administration of pituitary extracts. The result was confirmed by Guyenot, Ponse, Dottrens, Vallette and Trolliet (171) and Guyenot, Ponse and Wietrzykowska (173) using pituitary extracts, and by Guyenot, Ponse and Trolliet (172), Greene and Burrill (165) and Bradbury and Gaensbauer (27) using urine of pregnancy extracts. Greene and Burrill (166) also obtained evidence of stimulation in prostate tissue grafted into female rats injected with urine of pregnancy extracts. In view of the work of Lipschütz (260), Hill (192) and Deanesly (95) who showed that ovarian grafts into castrated males often caused marked development of the atrophic male accessory organs and that the effectiveness of the graft in this connection was proportional to the degree of theca luteinisation undergone by the graft, it was first supposed that the masculinising effect of the luteinised ovary in the female guinea pig was due wholly to the production of androgens by the lutein tissue. Guyenot and his collaborators, however, showed that the masculinising effect could be obtained in the ovariectomised animal by injecting pituitary extracts, but not by injecting urine of pregnancy extracts. The latter result was confirmed by Greene and Burrill (166) in their female rats carrying male prostate grafts. They conclude, therefore, that while the effect of the urine of pregnancy extracts is entirely mediated through the ovaries that of the anterior pituitary extracts depends partly or wholly on some other mechanism. Since the urine of preg-

nancy extracts used contained no other active principle than chorionic gonadotrophin, while the anterior pituitary extracts may well have contained adrenocorticotrophin, it may be surmised that the effect of the latter in ovariectomised animals was due to stimulation of the adrenals, leading to the secretion of physiologically effective amounts of androgen. The growth effect of oestrogens on the clitoris (Papanicolaou and Falk, 302; Turner and Burkhardt, 385), like that of the urine of pregnancy extracts, seems to be dependent on ovarian changes.

Gynaecogenic action in the female. The fact that in the usual course of events the ovariectomised animal does not show symptoms of oestrus even by the most delicate criteria known, complete vaginal cornification of the rat or mouse, precludes the possibility that the adrenals can have any considerable oestrogenic effect after removal of the ovaries. Nevertheless, phenomena highly suggestive of weak oestrogenic activity have been observed. Thus, it is well known that the time of opening of the vagina in the pubertal rat is only slightly affected by ovariectomy. According to Wade and Haselwood (388) removal of the adrenals alone has no more effect than removal of the ovaries, but removal of the adrenals as well as the ovaries may postpone opening for as much as two months. It seems, therefore, that ovaries and adrenals are alternative sources of the stimulus causing vaginal opening. This conclusion is similar to that arrived at by Burrill and Greene in relation to the early development of the female prostate in the rat. Other suggestive observations have been made about the behaviour of the reproductive tract after ovariectomy. Thus, Kostitch and Telebakovitch (240) found that even after complete ovariectomy there was a subdued rhythm in the vaginal epithelium of the mouse, a preponderance of mucus, leucocytes, or nucleated epithelial cells following each other in regular order; Frank, Goldberger and Salmon (138) and Geist and Salmon (153) noted that in some women after ovariectomy the vaginal contents and epithelium do not show the regressive changes usually associated with deprivation of oestrogen. There are also peculiarities in the response of ovariectomised animals to minimal doses of oestrogen. Tuisk (384) reported that in ovariectomised mice injected daily with small amounts of oestrogen the otherwise continuous vaginal cornification was occasionally, but irregularly, interrupted by invasions of leucocytes. A similar, but more precise observation was made by del Castillo and Calatroni (69) who found that vaginal cornification occurred periodically in ovariectomised rats injected with a constant low dose of oestrogen. Zuckerman (405) and Bourne and Zuckerman (25) confirmed these observations, and showed conclusively that whereas constant heavy dosage of oestrogen to the spayed rat led to persistent cornification, daily administration of a threshold dose led to the occurrence of cornification at intervals similar in length to the dioestrous period of the normal female. Di Paola (301) obtained analogous results with a daily dose of 0.1 μg. of oestradiol benzoate. The threshold dose varied in different rats, being between 0.5 μg. and 1.5μg. of oestrone. Zuckerman (406, 408) also showed that removal of the pituitary body did not abolish such artificial threshold cycles, but that in adrenalectomised rats maintained on desoxycortico-

sterone, the phenomenon could be reproduced only with the greatest difficulty. Zuckerman (404) observed a similar state of affairs in ovariectomised rhesus monkeys, in most of which the daily injection of 10 μg. of oestrone led to periodic bleeding such as can be produced by oestrogen withdrawal. This bleeding occurred at intervals of similar length to the normal intermenstrual period of the rhesus monkey, and indicated the occurrence of a periodic physiological drop in the oestrogenic stimulation given by a constant daily dose, caused either by a periodic decrease in sensitivity to oestrogen or a periodic withdrawal of an endogenous source. Recurrent menstruation-like bleeding has also been reported by Reynolds, Kaminester and Schloss (324) in an ovariectomised woman maintained on regular dosage of oestrogen. In continuation of the monkey experiments, Bourne and Zuckerman (26) have described morphological and histological changes in the adrenal of the rat associated with oestrus in the normal animal and with artificial oestrus in the ovariectomised rat injected with a threshold dose of oestrone. They concluded that the adrenal glands in the rat fluctuate in size in a cycle corresponding to the oestrous cycle, and that, though the changes are independent of both the pituitary body and the gonads, they exercise a direct effect on the accessory reproductive organs. It is not at present certain whether this cyclic response of the accessory reproductive organs to exogenous oestrogenic stimulation is due to the periodic production by the adrenal of small amounts of oestrogen, or, as suggested by Long and Zuckerman (266), periodic fluctuation in the adrenal controlled water metabolism causing fluctuations in the sensitivity of the accessory organs to oestrogenic stimulation.

In addition to the above observations, there is some evidence that under special conditions the adrenals may exert considerable oestrogenic activity in the ovariectomised animal. In this connection, the observations of Woolley, Fekete and Little (397) are of extreme interest. It is generally agreed that removal of the ovaries in the young animal largely prevents the appearance of mammary cancer even in the most susceptible strains. Woolley and his co-workers, however, found that if mice of the aba strain were ovariectomised at birth, more than 25 per cent developed mammary cancer at an average age of 522 days, as against about 50 per cent in untreated females. This result suggested strongly that considerable amounts of oestrogen ultimately appeared in the mice ovariectomised at a very early age, and this deduction was confirmed by the fact that the uterus, thin and anaemic six months after ovariectomy, was hypertrophied and hyperaemic in animals killed more than one year after operation, at which time several showed not merely vaginal cornification but cyclic appearance thereof. Woolley and his co-workers made careful examinations for regenerated ovarian tissue with negative results, but the adrenals were found to be enlarged and nodular, the degree of the hypertrophy increasing with increasing age. The experiments, taken in conjunction with the somewhat similar ones of Spiegel on male guinea pigs (see p. 227) undoubtedly suggest strongly that very early gonadectomy may result ultimately in highly effective androgenic or oestrogenic activity on the part of the adrenal.

Further progress could probably be made along these lines by the injection

of corticotrophic extracts into ovariectomised animals. A beginning in this direction was made by Moon (290) who obtained vaginal changes which he considered to indicate oestrogenic stimulation in immature ovariectomised rats injected with adrenocorticotrophic preparations. Further information was obtained by Nelson (295) who was able, by the injection of adrenocorticotrophic hormone, to cause enlargement of the uterus, vagina and mammary glands in gonadectomised or hypophysectomised, but not in adrenalectomised rats.

(b) *The adreno-genital syndrome.* It is not intended here to enter into a full discussion of the adreno-genital syndrome of which full reviews have been given from time to time by Broster and Vines (32), Grollman (169), Broster and his collaborators (31), Kepler (219), Rowntree and Ball (335) and Simpson (350). These together with the historical account given by Rolleston (333) should be consulted for details. The present brief account is intended solely to orientate the clinical observations on the adreno-genital syndrome as it occurs in man to the larger problem of the relationship between the adrenal cortex and the gonads in higher vertebrates.

The evidence discussed above suggests that in experimental animals the adrenals are capable, at least under certain circumstances, of producing oestrogens or androgens in physiologically effective amounts. According to present knowledge, however, spontaneous dysfunction of the adrenals on a scale adequate to cause disturbance of the reproductive organs and secondary sexual characters is seen only in man. The resulting condition, the adreno-genital syndrome, is of great interest, and in spite of the simplicity of the relevant biological conceptions, of considerable complexity. It is clear that the excessive production by the adrenals of gynaecogens in females, or of androgens in males, will lead to precocious or hypersexuality. The excessive production of the heterosexual hormones, on the other hand, will tend to cause inversion of the sexual organs and characters. The excessive production of both will lead to a complicated combination of precocity and inversion.

It is a general rule that the degree to which inversion of the reproductive organs can be produced depends on their degree of differentiation at the time when the inverting stimulus becomes operative. Thus, the effectiveness of any heterosexual adrenal activity depends on the age at which it begins, the earlier the more effective. It may be premised, therefore, that the clinical results of adrenal hyperactivity depends first on whether androgens or oestrogens or both are produced in excess, and whether the derangement appears prenatally, prepubertally, or in the adult.

The rôle of the foetal adrenal. In keeping with the above principles it has been suggested that prenatal abnormality of the adrenal cortex accounts for certain types of congenital hermaphroditism and that such types are the extreme expression of the adrenogenital syndrome. According to Broster *et al.* (31) "the common type of pseudohermaphroditism is a sex inversion of the female due to a cortical androgenic hyperfunction occurring before the end of the fifth month of foetal life". These authors consider that right up to the time of puberty sexual development is controlled by the adrenals, rather than by the gonads,

the former acting as bisexual accessory sex glands which are active throughout life and secrete both androgens and oestrogens, the more active gonadal mechanism normally being superimposed at puberty. This idea fits in, generally speaking, with some of the recent work on the effects of very early gonadectomy (see section Va, p. 224), but there seems no doubt that the gonads exert an influence on sexual development long before puberty. This aspect of the subject is far from clear-cut. It has been discussed in detail by Broster *et al.* (31), and also by Broster and Vines (32), Kepler (219) and Grollman (169).

The adreno-genital syndrome in children. Bullock and Sequeira (35) appear to have been the first to recognise that hyperactivity of the adrenal cortex may lead to changes of the sexual characters. Studying children under 15 years of age they observed the appearance of male characters in the females and the premature appearance of masculinity in the males in association with hyperplasia or neoplasm of the adrenal cortex. Further information and case descriptions of the condition as it appears in children were given by Glynn (158), Hoag(195) Collett (76), Gordon and Browder (162), Walters, Wilder and Kepler (389), Lukens and Palmer (268) and others. According to Grollman writing in 1936 the literature at that time recorded nineteen cases of the condition, of which five only were in boys. According to Reilly, Lisser and Hinman (323) writing in 1939, there were at that time forty verified cases of pseudosexual precocity of adrenal origin recorded in pre-adolescent girls, but as few of these records dated from later than 1936, the criteria accepted by these authors must obviously have been different from those used by Grollman. Looney (267), writing in 1940, stated that fourteen cases of the syndrome in boys, verified by operation or autopsy, had been recorded.

The abnormal changes usually begin during the first two years of life, and for the most part are those of masculinisation rather than of precocious puberty. In girls there is typically hypertrophy and protrusion of the labia, enlargement of the clitoris and growth of masculine facial and pubic hair, and sometimes deepening of the voice. The similarity of the changes to those which are associated with arrhenoblastoma of the ovary is highly suggestive (Novak and Long, 298). The condition of the ovaries and uterus does not seem to be constant. Grollman apparently discarded from his survey cases in which menstruation occurred, but other reviewers, such as Kepler (219) and Looney (267) accept premature menstruation as occurring sometimes in the typical syndrome seen in young girls. Reilly, Lisser and Hinman (323) consider it to be very rare. From our knowledge of the action of androgens on the female, the occurrence of premature menstruation would seem to imply gynaecogenic as well as androgenic action on the part of the tumour. However, such an idea is not discordant with the principles discussed above, and it is in keeping with the fact that Frank (137) found a high oestrogen content in the urine of several women with virilism. Whether or not premature menstruation occurs in the typical case the possibility of oestrogenic adrenal hyperfunction, which might lead, in a girl, to a condition of complete precocious puberty without signs of masculinisation, as described by Bennett (18), cannot be overlooked.

In boys showing the typical adreno-genital syndrome there is premature appearance of masculine hair, enlargement of the penis and external genitalia, and deepening of the voice. Grollman (169) states that the testis does not undergo development and does not show spermatogenesis, but Lisser (261) has reported to the contrary. The changes are generally similar to those described by Kunstadter (246) as having been produced in two boys by the administration of androgen (testosterone). In both sexes there is unusual muscular development, but this is more marked in the male, in which according to Harris and Plewes (183) it occurs in 60 per cent of cases. The fact that there is usually lack of testicular development shows that even in males the condition is not one of genuine precocious puberty and is distinguished thereby from the syndrome of pubertas praecox arising from tumours of the pineal. However, the extent to which androgenic stimulation may directly or indirectly affect spermatogenesis is not certain, and a condition closely resembling full precocious puberty cannot altogether be excluded as a theoretically possible consequence of adrenal hyper-function.

The essential association between the adrenal tumour or hyperplasia and the abnormal bodily changes is demonstrated by the fact that, in what is now quite a large number of cases in boys and girls, successful removal of the offending adrenal tissue has resulted in regression of the abnormal bodily changes. Striking examples of this effect have been recorded by Collett (76), Glynn and Hewetson (159), Walters, Wilders and Kepler (389), Lisser (261) and Lukens and Palmer (268). Recurrence of the tumour, or the further growth of metastases, results in a recrudescence of the bodily changes. Reilly, Lisser and Hinman (323) collected records of thirteen successful operations for the removal of the neoplastic or hyperplastic adrenal in cases of the syndrome in pre-adolescent girls.

Virilism in adolescents and women. In adults, masculinisation of women due to hyperplastic or neoplastic changes in the adrenals is a well defined syndrome, and full descriptions have been given by Glynn (158), Gallais (146) who gave the syndrome its present name, Glynn and Hewetson (159), Gordon Holmes (201), Fordyce (134) and Broster and Vines (32). It is evident from the reports cited by Kepler (219) and from the work of Goldzieher and Koster (160) and Broster and Vines (32) that the condition can occur as the result of simple adrenal hyperplasia as well as of neoplasia, and its essential connection with the adrenals rather than with the ovary is well shown by Kovacs' (241) report of the development of virilism after ovariectomy. The following description of the changes in adult women is given by Grollman (169): "The adreno-genital syndrome as it occurs in women is marked by an appearance of secondary male characters and a retrogression of the female sex characters. Among the former are (1) hypertrichiasis of the male type; (2) changes in bodily contour; (3) changes in the larynx; (4) hypertrophy of the clitoris, and (5) the assumption of the psychological outlook of the male. The retrogression of female characteristics is marked by a cessation of menstruation and the loss of other female characteristics which are replaced by the masculine characters already noted." These changes are

generally similar to those described by several authors as following the therapeutic administration of androgens to adult women. The ovaries of a woman from whom a large cortical malignant adenoma was removed were described by Rosset (334) as containing many primordial and Graafian follicles, but no corpora lutea. After successful operation there is re-feminisation with loss of hair, development of the breasts and return of menstrual function. According to Broster and Vines (32) and Goldzieher and Koster (160) the adrenal hyperfunction can be relieved by the removal of one gland, even when no tumour is present.

Adreno-genital syndrome in men. The adreno-genital syndrome rarely occurs in adult men, but the few reported cases are of great interest in demonstrating the ambisexual potentialities of the adrenals. From the fact that abnormal masculinisation is usually the chief symptom in boys, girls and women it might be supposed that adrenal hyperfunction in men would lead to super-masculinisation. Such a result, however, seems to be rare, though Macera (269) has reported such a case. The usual findings appear to be the reverse. Bittorf (20) and Holl (200) recorded feminism, testicular atrophy and gynaecomastia in men with cortical neoplasms. Holl's subject was of special interest in being the father of two sons, and in recovering from the gynaecomastia and other symptoms after the removal of the tumour. Holl also reported gynaecomastia in a boy with a very large adrenal tumour. Bittorf's case was further described by Mathias (282). Zum Busch (412) and Weber (392) reported a case of gynaecomastia and actual lactation in a man with a large "hypernephroma" and another similar one was recorded by Lisser (262). Simpson and Joll (352) have also reported the presence of a highly excessive amount of oestrogen in the urine of a man showing atrophy of the genitalia, impotence and gynaecomastia in association with a malignant adrenal tumour. The tumour was removed, causing a decrease in excretion of oestrogen, but later, with the growth of metastases which led to the patient's death, the amounts of oestrogen in the urine rose to as much as 6400 m.u. per litre. Biochemical observations on this case of considerable interest were reported by Burrows, Cook, Roe and Warren (43) (see p. 245). Two instances of testicular atrophy and gynaecomastia not associated with adrenal tumour, but thought to be due to adrenal hyperplasia, were described by Glass and Bergman (157). Neither of these patients excreted excessive amounts of oestrogen, but the androgen/oestrogen ratio was low for males. A case has also been described by Broster (30) in which feminism as defined by feminine body contours, small external genitalia and sparse pubic hair, was relieved by unilateral adrenalectomy after which the voice became deeper and the genitalia larger, and there was growth of secondary sexual hair.

In some of these cases the changes described as constituting feminisation, including female disposition of fat, do not seem to have been different from those which are seen in eunuchoidism, and which might have been expected to follow the atrophy of the testes. On the other hand, the latter symptom, and especially the gynaecomastia, must be considered highly significant. These changes, however, cannot be considered as occurring invariably in men with adrenal tumours (Crooke and Callow, 87).

Résumé of adreno-genital syndrome. If we accept the idea that the adrenals can secrete both androgens and oestrogens from the time they appear in the foetus right up into adult life, that these substances can affect the reproductive organs, and that hyperplastic or neoplastic changes in the adrenals may result in a greatly enhanced secretion of one or the other, then the theoretical possibilities emerge which are listed in table 1. Of these twelve types, the four (5, 7, 10, 11) in italics are well recognised and have been described above. Of the others, the hyper-isosexual adults (9, 12) would presumably not be very distinct types, the isosexual precocious girl (8) would be as described by Bennett (18), types 1 and 4 would be extreme cases of isosexuality, and the remaining types (2, 3, 6) would include various kinds of "hermaphrodite". According to Broster *et al.*,

TABLE 1

TIME OF OPERATION OF ADRENAL HYPERFUNCTION	GENETIC SEX	NATURE OF ADRENAL HYPERFUNCTION	
		Primarily androgenic	Primarily oestrogenic
Foetal	Male	1.	2. "Hermaphrodite". Femaleness superimposed on basically male organs
	Female	3. "Hermaphrodite". Maleness superimposed on basically female organs	
Prepubertal	Male	5. *Precociously masculinised boy.* ? Complete precocious puberty	6. Feminised boy
	Female	7. *Masculinised girl*, especially as regards secondary sexual characters	8. Precociously pubertal girl. Isosexual precocity
Adult	Male	9. Hyper-masculinised man	10. *Feminised man*
	Female	11. *Woman with virilism*	12. Hyper-feminised woman

2 and 6 are very rare owing to the lesser oestrogenic activity of the male adrenal and the greater stability of the male reproductive system.

Nature of the adrenal tissue undergoing hypertrophy. There has been much discussion of the precise nature of the adrenal tissue which undergoes hyperplastic or neoplastic changes, resulting in disturbance of the sex characters. The idea that some special tissue is involved is supported by the fact that neither hyperplasia nor neoplasia of the cortex necessarily leads to aberration of the sex characters, and that where such aberration occurs there is not usually any evidence that an excess of cortical hormones proper is being produced. Grollman (169) in this connection has called attention to the inner cortical layer which develops in the human foetus and undergoes involution after birth. This zone is probably represented in the adult by the thin layer of cells surrounding the medulla which

are readily distinguished from the cortex proper (Goormaghtigh, 161). Grollman (169) homologises this zone, under the name androgenic tissue, with the X-zone of the mouse. He considers that it is the seat of the masculinising tumours found in the human subject, and that malignancy of the cortex proper does not lead to any aberration of the sexual characters. Grollman's views, however, are not strengthened by his later conclusion (Gersh and Grollman, 155) that neither the "juvenile cells" of the rat, nor the X-zone of the mouse perform any androgenic function. In contrast to the idea that a special zone of tissue is involved, Broster and Vines (32) have put forward the view that there is present in the foetal adrenal of both sexes a special fuchsinophil substance which disappears at birth and appears post-natally in easily detectable amounts only when the adrenal undergoes hyperplasia or neoplasia of the type which leads to masculinisation.

In some instances, the offending tissue may apparently be outside the adrenal proper, as in the woman described by Saphir and Parker (337) who showed a typical clinical condition of adrenal virilism associated with apparently normal adrenals and aberrant adrenal tissue in the ovaries.

Relation with pituitary and other syndromes. It is outside the scope of the present review to consider the relation of the adreno-genital syndrome to pubertas praecox, Cushing's syndrome of basophilism, the Archard-Thiers syndrome, and the like. This subject has been dealt with in detail by a number of other writers, including Kepler (219), Grollman (169), Lescher (257), Simpson (350) Broster *et al.* (31) and Crooke and Callow (87). It must, however, be pointed out, that when aberrations of other endocrine organs, notably of the pituitary gland, are involved either primarily or secondarily, along with those of the adrenals, the effects on the reproductive organs and secondary sexual characters will be accompanied by a great variety of other bodily changes — obesity, plethora, etc. — which may render the subject totally abnormal quite apart from sexual peculiarities. For analogy it may be recalled that gonadal and thence changes in the other reproductive organs are only one of the many symptoms making up the syndrome of Simmonds' disease. It may also be remarked that the disturbance of the carbohydrate metabolism seen in the Archard-Thiers syndrome (diabetes of bearded women) is suggestive of over-production of the ordinary cortical hormones as well as of androgens by the neoplastic or hyperplastic adrenal.

The summary in table 2 is taken from Reilly, Lisser and Hinman (323).

(c) *Effects of cortical extracts and hormones on the accessory reproductive organs and secondary sexual characters. Cortical extracts.* Even before it was demonstrated that the adrenals might affect the reproductive organs of gonadectomised animals various kinds of adrenal extracts had been examined for activity similar to that of gonadal hormones. Thus, Müller (292) reported that he had been able to cause hypertrophy of the uterus of normal and thymectomised rats by the injection of protein-free cortical extracts. Asher and Klein (7) recorded similar results in females and also obtained hypertrophy of the reproductive organs in male rats. Klein (222) claimed that adrenal extracts accelerated the develop-

ment of the reproductive organs in male rats but inhibited it in female rats. Loewe, Marx, Rothschild and Voss (264) reported that an adrenal extract, not effective in cases of Addison's disease, caused hypertrophy of the rudimentary prostate in female rats. Observations have also been made in cases of Addison's disease receiving treatment with cortical extracts. Thus, Edwards, Shimkin and Shaver (116) noted swelling and tenderness of the breasts in a man after three months' treatment. There was recession of the glands after the end of treatment. According to Carr (65) and Hartman, Lockwood and Brownell (184) life-prolonging extracts of adrenals do not maintain lactation in adrenalectomised rats. When the characteristic cortical hormones were isolated, prepared in

TABLE 2

Relation of adreno-genital syndrome with other conditions

	TUMORS OF THE ADRENAL CORTEX	OTHER CONDITIONS CAUSING SIMILAR SYNDROMES
In intra-uterine life	Pseudohermaphroditismus	
In infancy		
Boy	Sexual precocity	Pineal tumor Testicular tumor Cerebral tumor
Girl	Pseudo-sexual precocity with pubic hair. Enlarged clitoris	True sexual precocity Adult genitalia Early menstruation Granulosa-cell tumor of the ovary
In adult life		
Male	Gynecomastia Femininization	Eunuchism
Female	Virilism or hirsutism Masculinization	Cushing's disease Oat-cell tumor of thymus Arrhenoblastoma of ovary

pure form, and their close chemical relation to the gonad hormones shown, immediate investigation was made as to whether they possessed similar biological properties. Only desoxycorticosterone has so far been available in amounts allowing of extensive tests.

Androgenic activity of desoxycorticosterone. Hooker and Collins (202) reported that desoxycorticosterone, in a daily dose of 20 mgm., caused a significant growth of the comb of the capon, and, in a daily dose of 0.5 mgm., stimulated the seminal vesicles and prostate gland of castrated mice, and the seminal vesicles but not the prostate of castrated rats. Hooker and Collin's mammalian experiments were not very convincing, and the results were contrary to those of Greene and Burrill (167) who made a careful histological study of the effects of desoxycorticosterone on the ventral prostate of castrated and castrated-ad-

renalectomised rats, and came to the conclusion that the substance had no demonstrable androgenic activity by this test, the most sensitive mammalian one known. Negative results on rats and chicks were also obtained by Paschkis (306). Chamorro (71) on the other hand found that administration of desoxycorticosterone delayed the atrophy of the accessory reproductive organs of the male mouse after hypophysectomy. Less direct evidence of androgenic activity has been brought forward by Selye (347) who showed that desoxycorticosterone causes regression of the X-zone of the mouse adrenal, and by Burdick and Konanz (36) who reported that the action of desoxycorticosterone on the uterus and blastocysts in early pregnancy is similar to that of testosterone. According to Dantchakoff (91) administration of desoxycorticosterone acetate to the embryonic or newborn rat does not affect sexual development. When combined with testosterone, however, it produces precocious development of the mammary gland and augments the effects of the androgen on the genitalia of the female. On the basis of present information it is extremely unlikely that desoxycorticosterone is a sufficiently good androgen to account for the observed androgenic activity of the adrenal. Probably the same applies to corticosterone, since the addition of the 11-hydroxy group is not likely to promote androgenic activity.

Oestrogenic activity of desoxycorticosterone. According to Salmon (336) administration of desoxycorticosterone in doses of 40 mgm. to 60 mgm. to post-menopausal women produces changes in the vaginal epithelium similar to those produced by oestrogens. Speert (359) also obtained results which suggest that desoxycorticosterone is oestrogenic in rhesus monkeys to the extent of increasing vaginal desquamation, causing sexual coloration and stimulating lobule-alveolar growth in the mammary gland. He found that bleeding of the oestrogen-withdrawal type followed cessation of injection.

Progestational activity of desoxycorticosterone. Desoxycorticosterone is chemically more similar to progesterone than to any of the other gonadal hormones, differing from it only in having a CH_2OH group in place of the CH_3 in position 21. This similarity is paralleled by the fact that progesterone has certain corticoid properties (see p. 224) and that desoxycorticosterone has been shown to evoke response in the typical tests for progestational activity. Miescher, Fischer and Tschopp (287) found that 10 mgm. of the substance evoked a typical response in the uterus of the sensitized immature rabbit (Clauberg test for progesterone). Similarly, de Fremery and Spanhoff (141) found, by the same technique, that 15 mgm. of desoxycorticosterone evoked about the same response as 2 to 3 mgm. of progesterone. Heuverswyn, Collins, Williams and Gardner (190) confirmed these results and added the interesting information that the adrenal substance, like progesterone, is able to potentiate the mating response to oestrogens in the spayed guinea pig, 10 mgm. being about equal in effect to 1 mgm. of progesterone. Torstveit and Mellish (383) obtained a similar potentiation in the guinea pig with both desoxycorticosterone and cortical extracts. Robson (328), in a more extensive study, showed that desoxycorticosterone also resembled progesterone in maintaining pregnancy in spayed mice and in spayed and hypophysectomised rabbits, in modifying the response of the rabbit's uterus to pituitrin, and in

suppressing the oestrous cycle in mice. Zuckerman (409), investigating the question in primates, found that large doses, 15 to 40 mgm. daily of desoxycorticosterone, like progesterone, inhibited oestrogen-withdrawal bleeding in the monkey. In only one of three monkeys, however, was there a typical progestational proliferation of the uterine endometrium; 1120 mgm. had been administered to this animal.

(d) *Presence of gonadal hormones and analogous substances in the adrenals.* The first impressive indication that the adrenal contained appreciable amounts of substances with an action similar to that of the gonadal hormones was provided by Engelhart (125, 126, 127, 128) who showed that crude lipoid extracts possessed both oestrogenic and progestational activity in tests on ovariectomised rats and immature rabbits. These observations were confirmed by Callow and Parkes (60) who further showed that extracts from horse adrenals were the most active and that the two activities could be fractionated by the method introduced by Allen and Meyer (3) for separating oestrogen and progesterone in extracts of corpus luteum. These authors found that 200 grams of fresh horse adrenal contained progestational activity equivalent to that of 1 mgm. of progesterone. They also showed that both activities were present in fractions of adrenal extracts discarded in the course of the industrial manufacture of cortin and it therefore seemed unlikely that the oestrogenic and progestational activity present in the adrenals had any essential connection with the characteristic cortical hormones. Further observations were made by Fischer and Engel (131) who reported that the adrenals contained 33 units of oestrogen and 5 units of progestational activity per kilogram. This work was brought to an early and highly satisfactory conclusion by Beall, who isolated both progesterone and oestrone in pure form from extracts of adrenal. The isolation of progesterone and incidentally of allopregnanolone, was carried out almost simultaneously by Beall and by Reichstein, and was the subject of a joint note (Beall and Reichstein, 17). In a further paper Beall (13) gave details of the chemical steps and identification, and showed that progesterone probably accounted for the whole of the progestational activity of the original extract. Later Beall (14, 16) reported the isolation of oestrone from each of two batches of adrenal material discarded in the manufacture of cortin. The hormone was separated from the phenolic ketonic fraction as its benzoate or dinitrobenzoate. The phenolic non-ketonic fraction was also oestrogenic, so that the whole of the oestrogenic activity of adrenal extracts is not due to the presence of oestrone. It is likely, however, that the active substance of the phenolic non-ketonic fraction is oestradiol or oestriol or both, as in the case of extracts of horse testis (Beall, 15).

As regards the androgenic activity of the adrenals, the situation is less clear-cut. Crude extracts of ox adrenal were found by Parkes (304) to have a slight androgenic activity as tested on the capon comb by the sensitive unction technique, but Carnes (63) failed to obtain a similar result with acetone-ether extracts of human foetal adrenal. Hodler (196, 197) claims to have produced stimulation of the prostate and seminal vesicles of castrated guinea pigs by the administration of alkali extracts of ox adrenals, the action being similar to that

of androgens. So far, however, no highly active androgen has been isolated from adrenal extracts. Reichstein (322) isolated a weak androgen, adrenosterone, which by virtue of a 15-keto group stands rather apart from the regular series of androgens. It is uncertain whether this substance can account for the whole of the observed androgenic activity of adrenal extracts.

It is impossible to say at the present stage whether the gonad-like activity of the adrenals in the gonadectomised animal is due to the secretion into the circulation of oestrone, progesterone and adrenosterone. It may, however, be noted that evidence obtained from the experimental animal suggests that appreciable androgenic activity may be exerted, while evidence of progesterone-like activity is missing. In adrenal extracts, by contrast, progesterone is relatively abundant, while androgens are weak and scarce.

(e) *Excretion of androgens and oestrogens after gonadectomy and in adrenal dysfunction.* It is well known that in man and certain other mammals, the urine of both sexes contains appreciable amounts of androgenic and oestrogenic activity. It was originally believed, without any real evidence, that the substances responsible for these activities originated in the gonads. It is now clear, however, that at least part of the substances must be of extra-gonadal, and is probably of adrenal origin.

Androgens and oestrogens of animal's urine. Little is as yet known about the excretion of androgens and oestrogens by laboratory animals. Smelser (355) and Schmidt (344) observed that detectable amounts of oestrogen were present in the urine of oestrous guinea pigs, and Emmens (private communication) finds that young female guinea pigs excrete about 0.2 I.U. per day in the urine; males excrete a similar amount. According to Emmens, male rats excrete much less than guinea pigs, about 0.005 I.U. per day. Koch (226) reported that rat urine contains less than one I.U. of androgen per litre. The source of the oestrogens in rat and guinea pig urine does not seem to have been ascertained, but the lack of striking sex difference suggests that the gonads are not the primary source.

A somewhat similar condition is found in monkeys. According to Allen, Diddle, Burford and Alder (1) chimpanzees excrete 49 to 142 mouse units of oestrogen in 24 hours, there being some cyclical variation during the menstrual cycle. Dorfman and van Wagenen (112) found up to 2.5 I.U. of oestrogen and up to 4.7 I.U. of androgen per 24 hours in the urine of male rhesus monkeys. These authors also found both substances in female monkeys. Fish, Young and Dorfman (132) obtained up to 13.5 I.U. per 23 hours of androgen and up to 135 I.U. of oestrogen from the urine of male chimpanzees. Figures obtained for oestrogens by these workers were similar to, though rather higher than, those given by Allen *et al.* Small amounts of androgens were found during the follicular phase of the cycle in females, but not during the luteal phase, though the authors do not attach significance to this on the data available. In general, they found that male chimpanzees excreted about twice as much androgen as females and somewhat less oestrogen. Highly significant experiments have been reported by Dorfman and van Wagenen (113). These authors found a great

increase in urinary oestrogens and a considerable increase in urinary androgens during pregnancy in the rhesus monkey. After removal of the foetus and ovaries no significant change in oestrogen or androgen excretion occurred. With the subsequent expulsion of the placenta, oestrogen excretion dropped to the non-pregnancy level or slightly below, while androgen excretion showed little change for about a month. From these most interesting experiments it may be concluded that, in the monkey, the greater part of the oestrogens excreted during pregnancy are of placental origin, but that the androgens, like the residual oestrogens, are derived neither from the placenta nor the ovaries and are probably of adrenal origin.

More is known about the urine of farm animals. According to Koch (226) bull and ram urine contains less than one I.U. and 4 I.U. of androgen per 24 hours. In large-scale chemical studies Marker (270) obtained androsterone, dehydro*iso*androsterone and oestrone from bulls' urine. He also (271) found the same three substances in steers' urine, in about the same amounts. According to Nibler (296) non-pregnant cows excrete small amounts of oestrogen, but data are not available on androgens, or for ovariectomised cows. Zondek's (403) remarkable observations showed that the stallion excretes large quantities of oestrogen, afterwards found to be largely or exclusively oestrone. By contrast, the urine of the gelding has little oestrogenic activity. It seems, therefore, that the androgens and oestrogens of bulls' urine are not of gonadal origin and are probably derived from adrenal secretions, while the oestrogen of stallions' urine seems definitely to be of testicular origin.

Androgens of human urine. It is unnecessary here to deal with the literature on this subject in detail; the review by Callow (58) may be consulted for references, etc. It will suffice to say that there is but little differentiation in the amounts of activity present in the urine of men and non-pregnant women, as first shown for androgens by Womack and Koch (396), and the active substances present appear to be the same. Thus, Dingemanse, Borchardt and Laqueur (103) found 40 to 50 units of androgen per litre of men's urine and 30 to 60 units per litre of women's urine. Callow (57) found 29 and 26 units respectively, and Gallagher, Peterson, Dorfman, Kenyon and Koch (145) 63 to 68 units and 42 to 56 units. Similar observations have been made by other workers. The work of Butenandt and Dannenbaum (44), Butenandt, Dannenbaum, Hanisch and Kudezus (45), and Butenandt and Tscherning (46) showed that the androgenic activity of male urine was mainly or wholly accounted for by the presence of androsterone, dehydroandrosterone and *iso*androsterone. After a detailed investigation, Callow and Callow (51, 52, 53) reported that androsterone and dehydroandrosterone occurred in the urine of normal women in amounts similar to those found in men's urine. The actual amounts were:—androsterone: men 1.6 mgm. per litre, women 1.3 mgm. per litre; *trans*dehydroandrosterone: men 0.2 mgm. per litre, women 0.2 mgm. per litre. They also obtained the inactive but related aetiocholanolone in similar amounts from the two sources. *Iso*androsterone was found in female urine by Pearlman (307). According to Nathanson, Towne and Aub (294) both boys and girls excrete similar small

amounts of 17-ketosteroids in early childhood; the amount increases with age, but more rapidly in boys.

This lack of quantitative or qualitative sex differentiation in the excretion of androgens throws grave suspicion on the idea that they originate in the gonads, and the suspicion is strengthened by the fact that hypo- or hypergonadal activity does not seem to have any great effect on the amount excreted. In the extreme case of gonadectomy substantial amounts of androgen are found in the urine. The literature is reviewed by Callow, Callow and Emmens (54). Initial failures to obtain androgens from the urine of eunuchs and ovariectomised women were probably due to deficiency of technique. Positive results were, however, reported by Koch (224, 225), Kenyon, Gallagher, Peterson, Dorfman and Koch (218), Hansen (181, 182), McCullagh (283) and Hamblen, Ross, Cuyler, Baptist and Ashley (177). The highest value obtained by any of these workers was 22 I.U. per litre for eunuchs, and 61 I.U. per litre for ovariectomised women. Callow, Callow and Emmens (54) found androgens without exception in the urine of a series of 11 eunuchs and 18 ovariectomised women. They found a maximum androgenic activity of 28 I.U. per litre for eunuchs, and 59 I.U. per litre for ovariectomised women, and they concluded that the average amounts were below those for normal men and women, but that individual figures came within the normal range. From the urine of a eunuch Callow and Callow (53) obtained androsterone 0.5 mgm. per litre, aetiocholanolone 0.9 mgm. per litre, and *trans*dehydroandrosterone 2 mgm. per litre. The first two were thus present in smaller amounts than in normal men (see above), the third in considerably greater amount. Hirschmann (194) obtained the same three substances from the urine of ovariectomised women in amounts but little less than those present in the urine of normal women.

These results make it extremely unlikely that the androgens and related substances of the urine of normal men and women are exclusively of gonadal origin. It remains now to consider the evidence that they are of adrenal origin. The evidence, as in the case of the gonads, is derived from cases of hyper- and hypofunction. Callow, Callow and Emmens (54) found that in the case of Addison's disease, as with gonadal deficiency, the androgen excretion was on the whole low, but that many values were within the normal range. The authors point out, however, that the patients were receiving cortical extract, which may well have increased the amount of androgen excreted. The substances present have not been identified beyond the fact that they are probably 17-ketosteroids. Much more dramatic results have been obtained in cases of adrenal hyperplasia. Simpson (349) first reported that the urine of a case of virilism contained an excess of androgenic substance, and suggested that it might be of adrenal origin. Later, Simpson, de Fremery and Macbeth (351) recorded the results of the examination of the urines from a series of cases of virilism and pseudohermaphroditism. They found up to 400 I.U. of androgen per litre, and gave definite form to the idea that in certain circumstances the adrenals might produce physiologically effective amounts of androgens. Callow (56) reported the isolation of 110 mgm. per litre of *trans*dehydroandrosterone from the urine of a girl

of six with a large adrenal tumour. In a more detailed report on the case Crooke and Callow (87) recorded a maximum excretion of 850 mgm. per litre of 17-ketosteroids, of which *trans*dehydroandrosterone was the chief constituent. In the case of a man with an adrenal tumour Crooke and Callow record a 17-ketosteroid value of 64 mgm. per litre. After removal of the tumour the excretion of 17-ketosteroid and androgen fell to normal. Other reports of excessive excretion of androgens in cases of adrenal tumour have been made by Cahill, Loeb, Kurzrok, Stout and Smith (49) 480 I.U. per litre, Kenyon, Gallagher, Peterson, Dorfman and Koch (218) 480 I.U. per day, and Slott (354) 2200 I.U. per litre. Lukens and Palmer (268) reported 160 I.U. per day in the urine of a masculinised adolescent girl; the androgen content fell to a fraction of this amount following the successful removal of the tumour. Masculinisation is found where these large amounts are present in women. Moreover, virilism in women presumed to be of adrenal origin, but not associated with malignancy, is often associated with the excretion of abnormal amounts of androgens, as shown by Kenyon, Gallagher, Peterson, Dorfman and Koch (218), Glass and Bergmann (157), Broster, Allen, Vines, Patterson, Greenwood, Marrian and Butler (31), Dingemanse and Laqueur (104), and Callow (58). Koch (227) and Glass and Bergmann (157) have called attention to changes in the androgen-oestrogen ratio, caused either by increase of androgen or decrease of oestrogen. Three steroid substances were isolated from the urine of women with adrenal hyperplasia by Butler and Marrian (47, 48), namely, the weak androgen *iso*androsterone, and the inactive compounds, aetiocholanolone and pregnantriol, the second of which is more closely related to the cortical hormones than to the androgens or aetiocholanolone.

In relating these results to the adrenal-genital relationship it must be remembered that, even if androgens and similar substances found in the urine of normal and gonadectomised men and women are wholly of adrenal origin, there is no proof that they are secreted by the adrenal as such. They may well be excretion products of the cortical hormones proper; testosterone, for instance, is almost certainly excreted partly as androsterone and aetiocholanolone (Callow, 50), and there is nothing inherently improbable in the idea that the gonadal and cortical hormones have common excretion products. It is unlikely, however, that the great amount of *trans*dehydroandrosterone excreted in tumour cases can all be derived from cortical hormones, but there is no evidence as to the form in which the excess *trans*dehydroandrosterone leaves the adrenal. It may be significant that Slot (354) failed to find androgenic activity in the tissue of the tumour of the case excreting 2200 I.U. per litre. However, it seems clear that the excreted androgen ascribable to adrenal activity is produced by the adrenal or in other tissues or fluids from adrenal products, and the end result, so far as androgenic activity on the part of the adrenals is concerned, is therefore the same.

Oestrogens of human urine. Knowledge concerning the distribution, quantity and nature of excreted oestrogens is analogous with that for the androgens, but is much less complete owing to the greater difficulties of extraction and characteri-

zation, and the smallness of the amounts present. As with the androgens, there is little sex differentiation in the amounts of oestrogen present in human urine and it usually occurs in the urine of menopausal women whose ovaries are atrophic (Kurzrok, 247). The unexpected presence of oestrogens in male urine was observed more than 15 years ago by Laqueur, Dingemanse, Hart and de Jongh (251), but accurate comparison of the amounts present in the urine of men and women was only made possible by systematic work on the conditions necessary for the extraction of maximal amounts. (See Callow, Callow, Emmens and Stroud (55) for discussion.) Even now it is difficult to compare results obtained in different laboratories, and the only comparisons for male and female urines which will be mentioned here are those where the figures have been obtained simultaneously by the same author. According to Gallagher, Peterson, Dorfman, Kenyon and Koch (145) the average daily excretion of oestrogens of normal men amounts to 90–120 I.U., and of women to 180–360 I.U., there being in the latter some cyclic variation during the menstrual cycle. Borchardt, Dingemanse and Laqueur (22) found that by the best method of extraction the oestrogen content of the urine was 330 I.U. per litre for women and 160 I.U. per litre for men. Oesting and Webster (299), working on children, obtained up to 60 I.U. per litre for boys and up to 80 I.U. per litre for girls. Dorfman, Greulich and Solomon (111) found up to 95 I.U. per day and much less for girls. Callow, Callow, Emmens and Stroud (55) examined two batches of urine from each sex by the same technique and obtained values of 57 I.U. per litre and 86 I.U. per litre for the women's and 19 I.U. per litre and 27 I.U. per litre for the men's. According to Nathanson, Towne and Aub (294) both boys and girls of 3 to 7 years of age excrete small amounts of oestrogen. Subsequently, increased amounts appear, but the amount increases more rapidly in girls.

The nature of the oestrogen in women's urine is not definitely known, but according to Smith, Smith and Pincus (356) fractionation of the urine concentrate by the method used by Cohen and Marrian (75) for separating oestrone and oestriol, yields two fractions both of which were active, and it may be supposed therefore that both oestrone and oestriol are present in normal women's urine. Marker, Rohrmann, Wittle and Lawson (272) have isolated two isomeric hexahydro-oestradiols, which they consider to be reduction products of oestrone, from the urine of non-pregnant women. More is known about the oestrogens of male urine. Dorfman, Gallagher and Koch (110) on the basis of biological tests concluded that the oestrogens of human male urine included oestrone, and also another substance which was not oestriol. Confirmation of this view was provided by the notable work of Dingemanse, Laqueur and Mühlbock (105) who obtained 6 mgm. of crystalline oestrone from 17,000 litres of men's urine. They report, however, that only one-third to one-half of the total activity passes into the ketonic fraction, so that an oestrogen other than oestrone must be present.

The source of the oestrogen of normal urine is uncertain, but as with the androgens, gonadectomy does not cause its disappearance. Thus, Frank, Goldberger and Salmon (138) note the presence of almost normal amounts of

oestrogen in the urine of each of twelve ovariectomised women. Laroch, Simonnet and Huet (252) found varying amounts, and Eng (124) and Kemp and Pedersen-Bjergaard (217) small amounts of oestrogens in the urine of ovariectomised women. Callow, Callow and Emmens (54) obtained oestrogenic activity from the urine of every one of 16 ovariectomised women at periods from one month to 27 years after operation. In some cases the amount was very small, and in all except one it was below normal. The exceptional case showed the high figure of 100 I.U. per litre. The occurrence of oestrogens in the urine of eunuchs is also established. Thus, Bingel (19) observed larger amounts than are usual in normal men; Eng (124) and Hansen (180) found small amounts. Kenyon *et al.* (218) record 30 I.U. per day and 45 I.U. per day of oestrogen in the urine of two eunuchs. Similar amounts, 60 I.U., were found by Dingemanse, Borchardt and Laqueur (103). Callow, Callow and Emmens (54) obtained oestrogen from the urine of each of 11 eunuchs, though the amounts present were very small. These observations make it extremely unlikely that the excreted oestrogens are wholly of gonadal origin even when the gonads are present, though one cannot entirely dismiss the possibility that their presence after gonadectomy is due to compensatory hyperactivity of some other organ.

There is now some evidence of the participation of the adrenals in oestrogen excretion, derived from studying the effects of hypo- and hyper-activity. Frank (136, 137) reported the presence of large amounts of oestrogen in the urine of women with adrenal cortical carcinoma. The amounts 1000–10,000 mouse units per litre were far above those seen in normal women or in cases of adrenal adenoma and hyperplasia. Saphir and Parker (337) found 5000 mouse units of oestrogen in the urine of a woman with adrenal carcinoma. Similarly, Burrows, Cook, Roe and Warren (43) obtained large amounts of oestrogen from the urine of a male patient with a malignant tumour of the adrenal. The substance was not isolated, but the chemical and biological results were concordant with its being oestrone. As a contrast to these observations on hyperactivity of the adrenals, Callow, Callow and Emmens (54) noted that oestrogen excretion was low in cases of Addison's disease.

In other work, the androgen/oestrogen ratio has been considered rather than the gross amounts of each. Thus, Glass and Bergmann (157) observed that in 10 women showing signs of virilism the ratio of androgen to oestrogen was increased, while in two men with gynaecomastia it was decreased.

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PHLORHIZIN GLUCOSURIA

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Phlorhizin is a bitter glucoside, $C_{21}H_{24}O_{10} + 2H_2O$, derived from the root bark of apple, cherry, plum and pear trees. It is also designated phloridzin, phlorizin, phlorrizin. The name is derived from the Greek—phloios, bark; rhiza, root. It occurs as minute, white, or slightly pinkish crystals, of a silky texture, or as a pale, yellow, light crystalline powder, odorless, and having a bitter, but later a sweet, taste. It is sparingly soluble in cold, but freely soluble in hot water, from which it crystallizes on cooling. It is soluble in alcohol (1 part to 4) and sparingly soluble in ether. The solutions are levo-rotatory. At 100°C., it loses water, and at about 107°C., it melts. When heated to about 130°C., it becomes solid again and melts again at 170°C. At about 200°C. it assumes a red color, due to the formation of rufin. Boiled with dilute acids, it is converted into sugar (phlorose) and phloretin. Exposed to air in the presence of ammonia, it assumes a purple color. Cold concentrated sulfuric acid dissolves it to a yellow solution and at 25° to 50°C. the solution becomes red (108).

Phlorhizin was discovered in 1835 by the Belgian chemist, L. de Koninck, who used the material in the treatment of malaria, on the grounds that it was a bitter like other remedies which were effective in this disease. In 1839, Stas discovered that the material was a glucoside and could be broken down by acid hydrolysis into phloretin—a white crystalline compound, $C_{15}H_{14}O_5$, which is said to have febrifuge properties—and phlorose, subsequently proven to be identical with glucose.

Von Mering, in 1886, determined the important physiological property of phlorhizin—the production of a glucosuria when introduced into the animal body—and this initial discovery laid the groundwork for many worthwhile investigations.

The literature has been reviewed from time to time (21, 49, 80, 90, 91, 110), but no complete review in English has been forthcoming.

Dosage. Von Mering (97, 98) at first gave it to dogs in a mixture with ground meat. One gram of phlorhizin per kilogram caused 10 grams per cent of glucose to appear in the urine. He was also able to elicit a glucosuria in phosphorous-poisoned animals, and even in animals with the liver extirpated, he found 1 per cent glucose in the urine. Subcutaneous injection of an aqueous solution of the drug with a small amount of sodium bicarbonate added enabled him to reduce the dose and still prolong its effects. Moritz and Prausnitz (105) learned that the sugar excretion begins three hours following the first of three equal (3.3 grams) doses of phlorhizin at six hour intervals, and the effect is complete in 33 to 36 hours.

In 1895 Coolen (21) proposed the suspension of phlorhizin in olive oil, and this became the standard procedure of later investigators. One gram of the

drug is suspended in 7 cc. of olive oil, and the mixture is injected subcutaneously. As the studies continued the D:N ratio was accepted as the best measure of effectiveness of the drug. Knopf (69) studied the intensity of glucosuria by changing the amount and method of injecting the drug. One gram of phlorhizin every 8 hours produced a D:N ratio of 2.8 to 3.2 during a 10-day period but the ratio rose to 4.2 during the following days when the dosage was raised to 2 grams three times a day. Minkowski (99) demonstrated that 1 gram daily would produce and maintain a D:N ratio of about 2.8 in rabbits. Baer, Hockendorf, Ringer as reported by Lusk (90) obtained similar results. Goldring (51) gave phlorhizin by mouth in some experiments on man and found that large doses—approximately 400 mgm. per kilogram divided into 4 doses at 45 minute intervals—were inadequate to raise the glucose clearance to that of the xylose clearance. He concluded that the oral administration of the drug is much less efficient than intravenous injection. Chassis, Jolliffe and Smith (19) state that the minimal dosage (intravenous) of phlorhizin required in man to produce total phlorhizinization is found to be in the range of 10 to 20 mgm. per kilogram body weight.

As Lusk (91) has stated, the necessary dose for maintaining maximal phlorhizin glucosuria has never been determined but common laboratory practice calls for the injection of 1 gram per day in oil as directed by Coolen (21). In 1941 Weissberger (151) following a recommendation of Sealock, dissolved the phlorhizin in propylene glycol and used this solution for subcutaneous injection with excellent results. The substitution of propylene glycol for the olive oil may prove to be the method of choice of the future. It must be kept in mind that the drug must be pure if one is to obtain uniform results.

Determination of phlorhizin in body secretions and tissues. Moritz and Prausnitz (105) reported that the absorption of phlorhizin from the intestinal canal, when given per os, is complete. They concluded that the drug is excreted in the urine and that the excretion is complete within 2 days. Loewi (85), however, was able to produce glucosuria by feeding 5 grams of an alcohol extract of a phlorhizinized dog's feces to a second normal dog. Glässner (Lusk 90) produced glucosuria in a normal dog by giving blood from a phlorhizinized dog and extracts of liver and kidney were also found to be moderately effective in this respect. Lambrechts (72, 73, 74, 75) proposed an accurate spectrographic method for the determination of phlorhizin in the serum. He was of the opinion that the kidney was not the principal organ of excretion of the drug since it disappeared from the plasma even when the kidney vessels were ligated. He thought that the muscle could destroy or transform phlorhizin, and that this tissue might be the main site for the elimination of the injected drug.

Mechanism of action of phlorhizin. The attention of investigators was early directed towards the effect of phlorhizin on the kidneys because of the glucosuria which develops. However, this abnormality of glucose excretion also directed attention to organs concerned with carbohydrate metabolism such as the liver, pancreas, and other endocrine glands. Many theories have been advanced but none have been proved.

Von Mering (90) proposed his "Elimination Theory" on the basis of the characteristic hypoglycemia that developed and he thought that the elimination of sugar depended on increased permeability of the kidneys for sugar and the excretion took place in the presence of normal or subnormal blood sugar concentrations. He believed that phlorhizin glucosuria was caused by a specific effect of the glucoside on the kidney.

Zuntz (170) first showed that phlorhizin has a direct effect on the kidneys. He inserted cannulas into the ureters of narcotized dogs, and watched the change in sugar concentrations in the urine from the individual kidneys after the injection of phlorhizin into one renal artery. He believed that the glucoside altered the normal retentive power of the kidney epithelium, and the glucosuria was due to secretion of sugar by the kidney cells. This concept was accepted by Erlandsen (40) and other workers of that period.

Levene (82) proposed that the glucosuria was due to increased production of sugar and not a diminished consumption of sugar within the animal, and that the proteins of the body and blood were the source of the extra sugar. This idea of overproduction rather than mere elimination was substantiated in his opinion as he found more sugar in the renal vein than artery, and the quantity of sugar in the kidney tissue increased after phlorhizin injection.

Minkowski (99) advanced his "Vehicle Theory" and expressed it by the equation: Phlorhizin $\rightleftarrows$ Phloretin + Phlorose. He believed that this breakdown occurred in the kidney. The phlorose (glucose) was eliminated while the phloretin combined with sugar in the blood, and then the new formed phlorhizin split once more in the kidney, etc. This theory was shown to be untenable by Lusk (90) and Charlier (18).

Loewi (85) next proposed the colloidal theory, that is, that part of the sugar in the blood is free and part is bound to a colloid. He considered that in diabetes mellitus, the extra sugar could not be bound by the colloid and was pas sively excreted through the kidney. In phlorhizin glucosuria, however, the kidney epithelium due to the influence of phlorhizin is enabled to break the colloidal link and eliminate the blood sugar in the absence of hyperglycemia. Stiles and Lusk (143) while accepting this theory added the hypothesis that the colloid sugar cannot be burned. Rosenfeld and Asher (129), Rona and Michelis, and Cammidge (as quoted by John, 66) all demonstrated that the sugar of the blood is in the free state and consequently Loewi's theory is untenable.

Lusk (90) quotes Mosberg and Nussbaum whose experiments were the first to indicate that the action of phlorhizin is on the tubular cells. They showed that the glomeruli of the frog's kidney were nourished by the renal artery, and the tubules by the reno-portal vein. Intravenous injection of phlorhizin was followed by glucosuria even though the glomerular action was cut off by ligature of the renal arteries. Injection of dextrose solution after ligature of the renal arteries produced no glucosuria in the frog until phlorhizin was given, whereupon glucose appeared in the urine. In the normal frog intravenous injection of dextrose produced an immediate glucosuria through the glomeruli. Nishi (Lusk, 90) believed that in phlorhizin glucosuria the sugar secretion took

place in the descending parts of the uriniferous tubules rather than through the glomeruli as in other types of glucosuria.

Pavy, Brodie and Siau (116) confirmed that no hyperglycemia develops following removal of the kidneys in phlorhizin glucosuria, and confirmed also the work of Zuntz. In their opinion the blood alone gave up sugar to the urine and attributed the glucosuria to an active formation of sugar by a catabolic action of the secretory cells of the uriniferous tubules. The kidneys split the sugar from a closed protein union (Loewi's colloidal theory) and then broke down the protein molecule itself after separation into the nitrogenous compound and sugar. It was assumed that the protein also contained a carbohydrate molecule in its structure. In opposition Coolen (21) found in phlorhizinized rabbits a hyperglycemia which was augmented by extirpation of the kidneys. He did not believe the glucuresis was of renal origin. Erlandsen (40, 41) reported that glucose excretion occurred at normal blood sugar levels and attributed the glucosuria to passage of phlorhizin through the kidneys and characterized it as an actual secretion. He believed that extra-renal effect of the drug had no foundation.

Nash (107) stated there is no doubt about the characteristic hypoglycemia which accompanies phlorhizin glucosuria. Since he found that the renal venous blood in phlorhizinized dogs shows a lower concentration of sugar than the general arterial blood he cast his vote for an increased permeability of the renal epithelium as a cause for the glucosuria. De Boer and Verney (30) agreed with Nash and could not uphold the work of Pavy et al. They used a heart-lung-kidney perfusion preparation and found that the amount of sugar appearing in the urine after addition of phlorhizin to the circulating blood could be completely accounted for by the fall in blood sugar over the same period of time. These results discourage the theory giving sugar producing powers to the kidney.

Häusler (55) postulated that the normal reabsorption of glucose by the tubules is arrested under the influence of phlorhizin and that the impermeability established for glucose normally in the direction of the vessel to the lumen of the urinary tubule is partly reduced.

White (158, 159) presented evidence to show that the action of phlorhizin was one which inhibited the tubules' power to absorb sugar and excited them to excrete sugar. Following this reasoning, Poulsson (119) studied the effects of phlorhizin by comparing the amount of sugar and creatinine, a "no threshold" substance, filtered into the urine, and had to conclude that phlorhizin completely paralyzed the ability of the renal tubules to reabsorb sugar. Shannon et al. (135), Shannon and Smith (137), Shannon (136) and Kuhlmann and Deviller (69) performed similar "non-threshold" substance experiments and came to the same conclusion.

Walker and Hudson (149) and Walker et al. (148) demonstrated that the greatest amount of glucose reabsorption takes place in the proximal half of the proximal tubule and that the proximal segment absorbs 80 per cent of the glomerular filtrate fluid. They were convinced phlorhizin affected the tubular

reabsorption capacity. Walker and Reisinger (150) could find no evidence for ascribing the glucosuria to any change other than the decrease in reabsorption from the tubules.

Smith (139) summarizes the kidney function aspect of phlorhizin by stating that "the administration of phlorhizin causes a marked reduction in all renal clearances, presumably due to circulatory disturbances, which fact makes it necessary to judge its effects in terms of clearance ratios rather than absolute values. Phlorhizin produces glycuresis by blocking reabsorption of glucose in all animals. It similarly blocks the reabsorption of xylose and sucrose, the reabsorption of which is slight and apparently due to entanglement with the reabsorption of glucose. The accompanying diuresis is due simply to osmotic resistance offered by the urinary glucose to the reabsorption of water."

Investigations appeared to have reached a standstill with little new work being done to forward the theories of phlorhizin mechanism, until 1933 when Lundsgaard (87, 88) reported his work and presented his phosphorylation theory. He stated that phlorhizin exerted a pronounced arresting effect on phosphorylation and dephosphorylation. He believed that esterification played a decisive rôle in glucose reabsorption, and consequently elaborated his theory of obstruction of phosphorylation by phlorhizin to explain the glucosuria. He considered that the phlorhizin glucosuria was a pure renal phenomenon. Wertheimer (156) verified that selective reabsorption of sugar from the intestines is impeded by phlorhizin and was in accord with reports of Cori (23), and Wilbrandt and Laszt (163). He concluded that the glucoside was a selective poison for the active sugar reabsorption in both kidneys and intestine.

Lambrechts (77, 78, 79, 80) was the first to criticize Lundsgaard's theory since he found, during phlorhizin glucosuria, no general inhibition of the processes dependent on the enzyme, phosphatase. Ellinger and Lambrechts (37) studied the excretion of various dyes in phlorhizinized frogs under the microscope and observed failure of reabsorption in the tubules. This demonstrated that substances other than glucose have their reabsorption affected by phlorhizin.

Anderson and Squires (4), Kutzler and Gutman (71), Weissberger (151), and Beck (8) all brought evidence to disprove Lundsgaard's theory. These papers deal with the study of phosphatase activity and phosphorus metabolism in normal and phlorhizinized animals. All are agreed that there is no evidence to indicate that phosphorylation is affected by phlorhizin. Lundsgaard finally abandoned the theory after he had performed additional experiments according to a report of Walker and Hudson (149).

In summary, none of the proposed theories of phlorhizin action have stood the test of time and experimentation. Von Mering's "elimination theory," Minkowski's "vehicle theory," Loewi's "colloid theory," theories of primary renal production of glucose and finally Lundsgaard's "phosphorylation theory" have all been proved to be untenable. Localization of the action of phlorhizin to the epithelial cells lining the proximal convoluted tubules seems fairly definite, and a mass of information concerning renal function has been gathered from these

investigations. The glucosuria would appear to be due to interference in reabsorption of the sugar by the kidney tubules, judging from the accumulated evidence.

Anatomical changes in the kidney produced by phlorhizin. Most of the important gross and microscopic changes due to phlorhizin have been recorded by the earlier investigators. Lusk (90) quotes the results of several workers. Junkersdorf reported hypertrophy of the kidneys if the organism is kept under the influence of the drug for a long period. They attain a size comparable to 0.66–1.15 per cent of the body weight; whereas the normal kidney is 0.4 per cent of the body weight. Pavy, Brodie and Siau (116) in contrast found no change in the volume of the kidneys. Trambusti and Nerti noted necrosis of the epithelium of the convoluted tubules in dogs which received 1 to 5 gram doses of phlorhizin per kilogram for 15 days. Von Kossa found severe cloudy swelling of the convoluted tubules of rabbit kidneys, and all the rabbits showed albuminuria. Policard and Garnier (117) reported hyaline degeneration of the convoluted tubules of white rats, but no changes in the remainder of the organ.

Phlorhizin effects in nephrectomized animals and those with ureteral ligation. Minkowski (99) noted that the hypoglycemia which developed in phlorhizinized dogs was abolished by extirpation of the kidneys and that the blood sugar remained normal even after the injection of more phlorhizin. Schabad (132) showed that ureteral ligation in a phlorhizinized dog caused no change in the blood sugar, whereas in a depancreatized animal marked elevation occurred. Csonka (28) observed that after ligation of renal vessels and ureters in a phlorhizinized dog, the subcutaneous or oral administration of 50 grams of dextrose caused an increase in the blood sugar from 58 up to 290 mgm. per cent in 2 hours. In normal dogs under otherwise duplicate conditions, the increases were only from 75 to 76 mgm. per cent.

Deuel, Wilson and Milhorat (33) reported that phlorhizinized nephrectomized animals, unlike diabetic animals, were found to be perfectly normal both in the height of the fasting R.Q., and in their response to small amounts of glucose given orally. This work showed that phlorhizin exerts no effect on the carbohydrate oxidizing mechanism.

Underhill (145) claimed a significant hyperglycemia developed when he gave phlorhizin to dogs which had the renal vessels ligated, or in rabbits in which kidney secretion was abolished as the result of subcutaneous injections of sodium tartrate.

Epstein and Baehr (39) found that in the absence of the kidneys, phlorhizin actually stimulated the accumulation of glycogen in the liver. The glucosuric effect of the glucoside is abolished as the result of nephrectomy.

Nephritis can diminish or entirely stop the sugar secretion in phlorhizin glucosuria. Schabad (132), Richter and Haberer (Lusk, 90) damaged the kidney by giving potassium dichromate and noted decrease in sugar excretion with finally cessation when the injury involved all of the organ. The normal excretory power of the tubules and of the glomeruli are lost by necrosis of the epithelium, and the phlorhizin, therefore, has no influence.

Effects of phlorhizin on liver. The rôle of the liver in carbohydrate metabolism and its influence on the course of phlorhizin glucosuria are reviewed by Sweet and Ringer (144). Extirpation of the liver in geese with subsequent phlorhizin injection causes no change in the glucosuria, whereas it is diminished in frogs (90). Rosenfeld (128) reported that the glucoside produced no glucosuria in Eck fistula dogs, but Sweet and Ringer found such dogs acted like normal dogs in every respect. Pick (Lusk, 90), and Ray, McDermott and Lusk (120) damaged the liver severely by sulphuric acid or by phosphorus and found no alteration in the glucosuria after phlorhizinization.

Influence on glycogen formation and destruction. According to Von Mering, glycogen persists in the liver of a phlorhizinized animal after a long fast, but Lusk (89) inferred that cold, exercise and adrenalin would mobilize such stores. Ringer (Nash, 110) found extra sugar in treated dogs following adrenalin administration, only if glycogen were present. Anesthesia and convulsions were effective in mobilizing the glycogen. Hoffman (60) was strongly against the idea that phlorhizin could mobilize glycogen from the tissues. Epstein and Baehr (39) found that the body accumulated glycogen in the presence of phlorhizin in the absence of the kidneys. Nash (109) studied dogs with ligated ureters and concluded that phlorhizin did not necessarily affect the glycogen in the liver or muscles. He states, however, that the phlorhizinized dog is able to synthesize and store extensively glycogen while in a state of hyperglycemia, and to a lesser extent even after deglycogenation and a continuing fasting hypoglycemia. Major (93) agreed that glycogenesis occurred when there was hyperglycemia. Schwartz and Sassler (134) reported data to indicate that the liver was disturbed by phlorhizin and that there was intereference in glycogenesis.

The relation of glycogen breakdown to production of glucosuria will be discussed later when the general problems of carbohydrate metabolism in phlorhizin poisoning are discussed.

Effects of phlorhizin on pancreas. Minkowski (99) demonstrated that the glucosuria from phlorhizin could not be due to an effect on the pancreas since the glucoside continued to produce its characteristic effect in depancreatized birds. Nash and Benedict (112) state that the theory of phlorhizin injury to the pancreas was not tenable since: *a*, the pancreas shows no alterations; *b*, Zuntz, classical experiment (s.v. p. 257); *c*, phlorhizin has the same effect on depancreatized dogs as on an otherwise normal dog.

Ringer (126) believed that the phlorhizinized dog had two incapacities, the first, an inability to utilize sugar, and second, an inability of the kidney to reabsorb the sugar that is filtered from the blood. He believed phlorhizin transitorially injured the pancreas since insulin when injected into a phlorhizinized dog caused oxidation of glucose and a concomitant storage of it as glycogen and reduction in the protein metabolism. Nash and Benedict (111, 112) felt that phlorhizin produced a real lesion in the sugar burning mechanism, but that it was not the result of injury to the pancreas as they were able to isolate insulin from the pancreas of maximally phlorhizinized dogs. Colwell (20) also disagreed with the ideas of Ringer.

Cori (22), Nash (108) and Möbius (104) agreed that insulin altered the picture in phlorhizinized animals. Cori noted a decrease in the sugar excretion although the nitrogen excretion was not diminished. Sugar was stored as glycogen which was later excreted when insulin was discontinued. He concluded that insulin did not directly influence sugar formation from proteins during phlorhizin poisoning. Nash reported that glucose and insulin caused abolition or decrease in ketosis and ketonuria, and improved the physical condition of the animal which later became one of extreme prostration when the glucose was discontinued. Extensive synthesis and storage of glycogen occurred in the muscles and liver. Möbius (104) found low glycogen in muscles and liver if phlorhizin was given alone but much higher glycogen contents if insulin was given also.

Dünner and Mecklenberg (36) claimed that insulin is unable to prevent even minute glucosuria brought on after small doses of phlorhizin. However, in diet controlled diabetes they noted a fall in R.Q. and the appearance of acetone if the glucoside was given. If insulin was given also the R.Q. rose again and the acetone disappeared. They interpreted these disturbances as due to insufficient sugar combustion in the tissues caused by the phlorhizin.

Allen (1) as a result of sugar tolerance studies concluded that phlorhizin does not injure the islets of Langerhans. Goldstein et al. (52, 53) analyzed glucose tolerance curves obtained from intact dogs and from dogs with the ureters ligated when insulin was given as well as phlorhizin. They concluded that insulin exerts its entire effect on the sugar utilization and that it in no way corrected the action of phlorhizin on the kidneys.

As a result of all of this work, it would appear that phlorhizin has its effects on the kidney and that no injury is caused to the pancreas.

Effects of phlorhizin on hypophysis. Houssay and his co-workers (11, 61, 62) noted that the phlorhizin glucosuria of a hypophysectomized dog was characterized by *a*, high mortality; *b*, elimination of glucose less actively; *c*, diminution of urinary nitrogen; and *d*, low D:N ratios. With a lesion of the tuber cinereum the glucosuria is identical with that of controls, indicating a dissociation of function of this part of the gland. Injection of extract of the anterior lobe of the pituitary in hypophysectomized dogs is beneficial since it *a*, prevents the hypoglycemia and death; *b*, increases the glucosuria to control levels; *c*, increases the diuresis and fall in weight; *d*, increases ketone body elimination; but *e*, does not always increase the urinary nitrogen.

Rietti (122) found that ketone excretion of phlorhizinized dogs is much less than that of normal controls and that pituitary insufficiency always diminished the urinary elimination of ketone bodies. Black (13) reported that rats treated with anterior pituitary extract containing the ketogenic principle became resistant to its action within 3 months and such animals showed only slight acetonuria when treated with large doses of phlorhizin while fasting.

Gaebler and Zimmerman (47) gave a large injection of the anterior pituitary growth hormone to a phlorhizinized dog on the fourth day and reported a weight increase and a smaller nitrogen output than in experiments with phlorhizin

alone. The effect of this preparation used above on nitrogen output was increased by thyroidectomy, presumably because the absence of the gland reduces the metabolic rate of phlorhizinized dogs to approximately normal levels. In thyroidectomized-depancreatized dogs receiving a constant amount of insulin, the same doses of the pituitary extract were severely diabetogenic.

Effects of phlorhizin on adrenals. Adrenalectomy in phlorhizinized rats greatly decreases sugar and nitrogen excretion according to Evans (43). Adrenal cortical extract does not alter the sugar or nitrogen excretion of adrenalectomized-phlorhizinized rats. Ketosis of glucoside treated rats is diminished by adrenalectomy. He concluded that the adrenal cortex is concerned in the conversion of protein to carbohydrate.

Robbers and Westenhoeffer (127) report that the glucosuria of phlorhizin is not influenced by adrenal cortical extract or corticosterone. Wells and Kendall (153) were able to increase the low rate of glucose excretion of the phlorhizinized, adrenalectomized rat by giving corticosterone and closely related compounds. The rate of gluconeogenesis of operated rats could be increased to that of normal phlorhizinized rats by the compound E (17 hydroxy 11 dehydrocorticosterone). This finding illustrates the importance of the adrenal cortex in carbohydrate metabolism.

Wells and Chapman (152) determined that in hypophysectomized-phlorhizinized rats the excretion of glucose and nitrogen was about half that found in adrenalectomized-phlorhizinized rats. Treatment of such rats with compound E or with corticosterone acetate markedly increased the rate of gluconeogenesis. If such rats were given compound E and thyrotropic hormone the rate of gluconeogenesis approached that of normal phlorhizinized rats. Thus, both the adrenal and thyroid glands affect the formation of glucose in hypophysectomized-phlorhizinized rats.

Wells and Kendall (154) state that results obtained when the adrenal medulla only is removed, indicate that the action of epinephrine is not significant. These workers conclude that the metabolism of exogenous protein is not seriously impaired by the absence of the adrenal cortical hormone. It is only where the exogenous supply is lacking and endogenous protein must be metabolized that the limited capacity of gluconeogenesis of the adrenalectomized animal becomes evident.

Effects of phlorhizin on thyroid Dann et al. (35) reported that thyroidectomy may lead to failure of increase in heat production usually observed after phlorhizin administration and protein metabolism is affected also. Wills and Kendall (153) noted that thyroidectomy diminished the amount of glucose and nitrogen excreted by phlorhizinized rats but that thyroxine restored the rate of gluconeogenesis.

The effects of phlorhizin on secretions of the breasts, skin glands, and on pancreatic, gastric and salivary secretion have been studied (26, 118, 90, 110). The most that can be reported is that the secretion may contain a reducing substance in very small amounts. Splenectomy does not effect phlorhizin glucosuria (5).

Influence of phlorhizin on body fluids and secretions. Von Mering noted that the composition of the urine of a phlorhizinized dog resembled that of a human diabetic. There was a high ammonia content, much acetone, and a strong levo-rotation after fermentation, indicating the presence of B-hydroxy-butyric acid. The dog finally became comatose from which state it could be aroused by giving meat and milk by stomach tube. He also noted that after giving phlorhizin to a starved dog the amount of nitrogen in the urine was increased, but this increase was not as large if fat were fed at the same time. Moritz and Prausnitz (105) discovered that the absolute amount of sugar excreted from meat and carbohydrate food depends on the ingested amount. As much and more sugar can be obtained from the meat as from carbohydrate if the meat is given in abundant proportions. Coolen (21) confirmed these findings.

Reilley, Nolan and Lusk (121) observed that a starved phlorhizinized dog excreted five times the amount of nitrogen as that excreted by an ordinary fasting dog. The maximal nitrogen excretion appeared on the second or third day of phlorhizinization and fell gradually.

Loewi (Lusk, 90) reported that diuretics in normal kidneys increased the secretion of sodium chloride and urea, but not P_2O_5 and if given during phlorhizin glucosuria there is an increase in the chloride secretion but not in the secretion of P_2O_5 or dextrose. He concluded that chlorides, urea, and sugar are easily filtered through the glomeruli, while P_2O_5 is freed from combination in the blood and preserved by the kidney.

Lusk (90) reported that urea nitrogen in a starved dog was 89 to 92 per cent of the total nitrogen. Excreted ammonia rose sharply and in one case reached a maximum on the second day of the glucosuria which was the same day the maximum acetone and B-hydroxy-butyric acid excretions occurred. Creatine nitrogen increases were also noted. Sulfur content rose proportionally to the rise of nitrogen, thus maintaining the ratio found in the fasting condition.

Mandel and Lusk (94) studied the carbon in the urine and reported that as much as 60 per cent of the phlorhizin carbon may be eliminated in the urine. In the early stages of phlorhizin glucosuria the carbon in the urine derived from oxybutyric and other abnormal products except sugar appears to be negligible.

Effects of phlorhizin on blood. The hypoglycemia first noted by Von Mering is the most important alteration in the blood of the phlorhizinized organism. Erlandsen (40) showed that the blood sugar level depended on the amount of glycogen in the liver available for use as glucose. De Boer and Verney (30) demonstrated that there was a direct relation between the amount of sugar appearing in the urine and the fall of the blood sugar during the same period.

Ets (42) found an acidosis, as indicated by the fall in alkali reserve and increase in H+ ion concentration, hypoglycemia, an increase in the blood N.P.N., and an increase in the blood lipid constituents.

Himwich et al. (58) observed that the liver of phlorhizinized dogs removed fat from the blood until the fifth day when the accumulated fat began to be liberated. In depancreatized dogs continuous accumulations of fat from the

blood were noted. They (59) also studied the blood in the portal and hepatic veins in phlorhizinized dogs and learned that acetone is added to the blood by the liver.

Levene (81) found decreased sugar in the lymph and also thought there was sugar in the bile as the result of phlorhizin injections. Ray, McDermott and Lusk (120) discovered the presence of bile salts but no traces of sugar in the vomitus of phosphorus poisoned phlorhizinized animals, but Woodyatt (165) reported evidence of glucose in bile.

Smyth and Whipple (140) determined that phlorhizin has no effect on bile salt output in bile fistula dogs.

The D:N ratio. An excellent review of the D:N ratio is given by Lusk (90) and the essential details only are given below. Minkowski found in his depancreatized dogs that the urine constantly contained dextrose and nitrogen in a ratio which approached 2.8 grams of dextrose to 1 gram of nitrogen. Since 1 gram of nitrogen is equivalent to 6.25 grams of muscle protein, it was calculated that in "total pancreatic diabetes," 45 per cent of the protein molecule was excreted in the urine as dextrose.

Lusk originated the term "extra sugar" to indicate the sugar which resulted from an ingested substance or from the carbohydrate catabolized in the organism itself, and not from simultaneous protein metabolism. The sugar contained in the glucoside phlorhizin does not affect the D:N ratio.

Cremer and Ritter after giving frequent doses of phlorhizin to fasting rabbits obtained ratios varying between 2.64 and 2.8 and considered such ratios to indicate "total phlorhizin diabetes." Reilley, Nolan and Lusk were unable to produce a ratio of 2.8 in dogs but established ratios in a range 3.38 to 3.91. Later corroboratory work confirmed such ratios and consequently the customary D:N ratio in a fasting phlorhizin dog is considered to be 3.6 to 3.7. Lower ratios are seen terminally in dogs which die of phlorhizin poisoning or some other pathological lesion.

On the basis of a 3.65:1 ratio of glucose to nitrogen, it is calculated that about 58 per cent of the protein molecule is convertible to sugar. Reilley, Nolan, and Lusk (121) concluded that when meat is fed, the sugar derived therefrom is quantitatively recovered in phlorhizin glucosuria, but it may be eliminated before the nitrogen belonging to it. Nash and Benedict (111) confirmed the different excretory rates of dextrose and nitrogen.

Influence of body glycogen. An especially high value for the D:N ratio will always be found in the urine of the first day of a fasting phlorhizinized dog. The excess of sugar is due to the loss of blood sugar and from mobilization of glycogen stores. Phlorhizin alone does not clean out all of the glycogen from the body, but cold, work or adrenalin administration during phlorhizinization are reported to cause complete freeing of the tissues of their glycogen stores. However, correct ratios of 3.6 to 3.7 may be obtained by fasting and phlorhizin administration over a period of 3 to 4 days.

Lusk (90) concluded as the result of many experiments that phlorhizinized

animals store up no glycogen and that when sugar is formed from ingested material, all of it will be eliminated and none stored as glycogen. He concluded also that sugar cannot be produced from fat.

Influence of carbohydrate feeding. Dextrose fed in small quantities per os or injected subcutaneously is not utilized but eliminated quantitatively. Phlorhizin glucosuria is considered to be a complete glucosuria and dextrose (within limits) cannot be utilized (143).

Influence of protein feeding. Reilly, Nolan and Lusk (90) analyzed the effect of meat feeding on the D:N ratio. They noted that after feeding 500 grams of meat the nitrogen and sugar excretion in the urine can be doubled while the D:N ratio remains unchanged. The sugar arising from the protein can be excreted more quickly than the corresponding nitrogen. Gelatin feeding also causes no change in the ratio but the protein metabolism of the body was decreased.

Influence of fat feeding. Fat feeding in phlorhizin glucosuria has no effect on the sugar excretion in the sense that the ingested fat is not changed into dextrose. Von Mering showed that fat ingestion reduced the nitrogen and sugar excretion in the urine. Reilley, Nolan and Lusk (90) established that fat feeding had no effect on the D:N ratio.

Acetone bodies. While the fasting dog shows almost no acidosis, the fasting phlorhizinized dog secretes acetone and B-hydroxy-butyric acid in the urine and coma finally develops.

Various investigators (48, 10, 160, 161) have demonstrated that meat or carbohydrate feeding to a phlorhizinized dog lessens the degree of acidosis with reduction in the amount of acetone and hydroxy-butyric acid. Study of the D:N ratio in the urine showed that the fall in acidosis cannot be accounted for by combustion of the sugar.

The acetone content of the blood, liver, kidneys and lungs from a phlorhizinized dog may be double the amount found in a normal dog, and it has been demonstrated that B-hydroxy-butyric acid is oxidized when given to a normal dog but very poorly oxidized by a phlorhizinized dog (90). Himwich et al. (59) by determinations on the portal and hepatic venous blood found that acetone was added to the blood by the liver. Feeding of the sodium salt of B-hydroxy-butyric acid does not effect any change in output of sugar by a phlorhizinized dog (106).

Soskin (141) postulates two theories of ketosis and the formation of the ketone bodies: *a*, that an inability of extra-hepatic tissues to dispose of the ketone bodies exists; *b*, a rate of hepatic production in excess of the existing normal rate of peripheral disposal is present. Nash (109) was of the opinion that the acidosis and ketosis which characterize phlorhizin glucosuria may result from the lowered concentration of carbohydrate in the tissue.

Mirsky (100) demonstrated that ketones arise only in the liver, and that insulin which suppresses ketosis does so by acting on the liver and not on the extra hepatic tissues. Evidence indicates that in diabetes (pancreatic) the ketone bodies, like the blood sugar, are utilized by the extra-hepatic tissues, but are produced by the liver at rates which exceed the power of the tissues to dispose

of them. Mirsky and Brohkahn (102) attributed the effect of glucose in ketosis to an inhibition of fat oxidation in the liver. Ketosis depends on the rate of ketone formation and the rate of ketone utilization.

Möbius (104) found that under the influence of insulin, the sugar and nitrogen excretion in the urine declines in the phlorhizinized fasting animal. Insulin prevents the ketonuria that is characteristically present.

According to Rietti (122) ketone elimination was less in pancreatic diabetes or hypophysectomized animals than in controls, and was related to the level of the blood sugar. In fed hypophysectomized dogs, the ketone elimination in phlorhizin glucosuria is always less than in corresponding controls. Sugar feeding diminishes the ketone excretion in the controls, but in hypophysectomized animals there is a slight rise. Ketone excretion of phlorhizinized-hypophysectomized animals does not increase as much as does the glucosuria on a meat diet.

Goldfarb and Himwich (50) studied the ketone substances in depancreatized and phlorhizinized dogs. In 8 dogs the heart removed acetone substances from the blood and in 3 cases added them, but the brain caused no changes in the blood. The testicles removed acetone in 6 cases and showed no change in 8 others. These results were interpreted as giving some indication of the character of the foodstuffs oxidized by the individual organs.

Total and intermediary metabolism. Experiments concerning the total energy metabolism of phlorhizinized animals are adequately covered in Lusk's review (90).

The rôle of phlorhizin glucosuria in the study of intermediary metabolism constitutes one of the most important aspects of scientific investigations with this drug. While phlorhizin glucosuria is essentially an interference with metabolism of carbohydrates, much new light has been thrown on the close interrelationship of the three major groups of nutrient material, carbohydrate, protein and fat, by the use of the drug.

Phlorhizin is not only the most convenient but the more effective means of inducing experimental diabetes, in respect to the degree of carbohydrate intolerance created. Early experiments on the carbohydrate intermediary metabolism are summarized by Lusk (90). When *levulose* or *galactose* is given to a completely phlorhizinized animal, some extra dextrose appears in the urine but no levulose or galactose is excreted. No lowering of the protein metabolism was noted (90, 121). When *lactose* is fed, Lusk reported that no lactose was present in the urine but the protein metabolism was markedly reduced, and the reabsorption and combustion of a large part of the lactose was demonstrated. Following the intravenous injection of *sucrose* the sucrose secretion increases without extra dextrose appearing in the urine. If an equimolecular solution of dextrose and sucrose are given intravenously, the phlorhizin effects a greater excretion of sucrose than of dextrose. Pentoses and cellulose caused no increase in dextrose excretion in the urine. Glycerin, lactic acid, and propyl alcohol are changed into dextrose in the phlorhizinized organism, while other alcohols and both mono- and di-basic fatty acids have no influence on the D:N ratio and are not converted into dextrose (90).

Baer and Blum (7) reported that subcutaneous injections of glutaric acid in

a phlorhizinized dog considerably reduced the dextrose, the nitrogen, and the B-oxybutyric acid of the urine. However, they also stated that glutamic acid, alanine, glycocol and lactic acid had no remarkable influence on the amount of excreted sugar, and it is well known that these substances are sugar formers. Lusk (90) considered that their animals were incompletely phlorhizinized and that conclusions drawn from the experiments were not conclusive.

Mandel and Lusk (95) noted that lactic acid disappears from the blood and urine in phosphorus poisoning when phlorhizin glucosuria is induced. They concluded that this indicates that lactic acid, produced from cleavage and denitrogenation of protein, is first synthesized to dextrose before distribution to the tissues.

Loebel et al. (84) found that lactic acid may be formed in an animal which has lost the power to oxidize carbohydrate and present this as evidence that the defect in phlorhizin glucosuria is not in the formation of lactic acid from carbohydrate. This work contradicts Embden (38) who suggested that carbohydrate could not be oxidized in phlorhizin glucosuria because it could not yield lactic acid. Loebel et al. hypothesized that during the contractile phase of muscular activity energy is supplied by the exothermic chemical reactions which result in the formation and neutralization of lactic acid. These reactions occur even during complete phlorhizin glucosuria and glycogen depletion. During the recovery phase the lactic acid is reconverted to glycogen and they thought that in phlorhizin glucosuria the chief, if not the only, source of energy during the recovery phase must be furnished from fat.

The conversion of succinic acid to glucose in the phlorhizinized dog is reported by McKay and Barnes (92) in the ratio of 2 mols succinic acid → 1 mol dextrose. When larger doses are given, a small percentage is excreted in the urine as sugar, but anti-ketogenic and nitrogen sparing activity indicate its conversion to dextrose before being burned.

Deuel and Chambers (32) determined the hourly rate of elimination following the oral administration of glucose, galactose, fructose and lactose in phlorhizinized dogs. Rate of excretion of fructose and galactose were practically identical with that of glucose itself, thus indicating that the intermediary transformations involved in the change of these monosaccharides to glucose is a very rapid one. Ninety-four per cent of the fructose and 80 per cent of the galactose was recovered as extra glucose but lactose was more slowly excreted and 50 per cent only was recovered in 2 hours as dextrose. Sparing action of nitrogen metabolism was noted despite the fact that none of the sugar was apparently burned. Muscular power of the glucoside treated animal was restored even though the glucose was quantitatively excreted.

The major problem in the intermediary metabolism of carbohydrate is *whether or not the glucose is actually utilized by the organism in the oxidative energy-producing process.*

Lusk (90) stated that if the phlorhizin glucosuria is a "total glucosuria," then the ingestion of dextrose must be followed by a complete excretion in the urine in the form of extra sugar. Stiles and Lusk (143) believed that within certain

limits the phlorhizinized dog had lost its power to oxidize dextrose but if carbohydrate is given in sufficient amounts, it can be oxidized.

Rosenfeld (129) gave a normal dog 100 grams of dextrose by vein and recovered 21 to 28 grams of extra sugar in the urine, whereas 36 grams appeared in the phlorhizinized dog. If the dextrose was fed, he recovered 78 grams of extra sugar. Lusk attributed these findings to the degree of concentration of dextrose in the blood stream. If intravenously given, the sugar concentration in the blood stream would be high and cells would obtain it in amounts sufficient for oxidation and less elimination would result. When fed, absorption would be slower and consequently concentration would be much less and so it was not oxidized and was recovered as such in the urine. Sweet and Ringer (144) express the findings of Rosenfeld in a formula, viz.: The amount of glucose that may be oxidized is a function of the ratio between the velocity of absorption of glucose and the velocity of its excretion by the kidneys.

Nash and Benedict (111), however, produced hyperglycemia, which was maintained for 6 hours or longer, but quantitatively recovered the sugar in the urine. They took the view that phlorhizin produces an intrinsic impairment of utilization of sugar by the tissues. Wierzuchowski (162) also considered that there was a general hindrance in glucose utilization since phlorhizin treated animals die as the result of hypoglycemia with convulsions or ketosis with coma. Both of these states are visible exponents of the extremely low level of carbohydrate metabolism in the body cells.

Deuel, Wilson and Milhorat (33) working with nephrectomized-phlorhizinized animals found that the nitrogen metabolism was like that of controls which received no phlorhizin. Acetone was not produced as in the ordinary animal, and blood sugar values were constant. The phlorhizinized nephrectomized dogs must have oxidized glucose normally. They suggested that two avenues of escape from oxidation are offered the ingested glucose. The first is that a large proportion is rapidly removed from the field of possible oxidation by excretion as extra sugar, and second, that it is stored as glycogen temporarily which is later excreted when the blood sugar falls. The evidence indicates that the action of phlorhizin is entirely a renal one. Carbohydrate is not oxidized during fasting or following ingestion of small amounts of glucose since the concentration of glucose in the blood and tissues is low and not due to any intrinsic impairment in the mechanism for its oxidation.

Boothby et al. (15) studied the respiratory quotient and D:N ratio in phlorhizinized dogs and discovered that the basal metabolic rate is markedly elevated and may reach 90 per cent above the normal value. Administration of glucose causes a definite decrease in the high level of heat production and a definite rise in the R.Q. If the rise in R.Q. is taken as indicating an increase in the oxidation of glucose, it is found that 18 to 25 per cent of the ingested glucose is oxidized in 5 to 7 hours.

Deuel (31) also presented evidence indicating that there is no intrinsic impairment of the ability to oxidize carbohydrate existing in the phlorhizinized animal but that phlorhizin action on the kidneys results in mobilization and

drainage of available glucose. The carbohydrate ceases to be oxidized because of the low concentration in the blood and tissues and there is no stimulus for insulin production. Acidosis ensues and the glucose tolerance is reduced. The glucose tolerance curves of the phlorhizinized animals are distinctly of the diabetic type.

Drury, Bergman and Greely (34) studied phlorhizinized hepatectomized dogs and determined that 75 mgm. per hour of glucose are required to maintain such an animal. As a result of their experiments, they were convinced that the tissues of such glucoside treated dogs do utilize sugar even though the blood sugar level is kept low. Bollman, Mann and Magath (14) concluded that the energy turnover of the liverless animal does not come from protein but rather from the *direct utilization of fat.*

Soskin, Levine and Lehmann (142) concluded also that there is no diminution in the utilization of carbohydrates by phlorhizinized dogs as compared with normal dogs. They believe the low R.Q. in phlorhizin glucosuria is due to overproduction rather than under-utilization of sugar. The low R.Q. results from a predponderance of reaction in which oxygen is used up without the corresponding production of carbon dioxide, such as the formation of sugar from protein and fatty acids. The excessive gluconeogenesis in both phlorhizin glucosuria and pancreatic diabetes, although caused by different mechanisms, nevertheless lead to similar consequences, namely, continued glucosuria, low liver glycogen values, accumulation of fat in the liver, ketosis and a lowered R.Q.

It is therefore apparent that the D:N ratio of phlorhizin treated animals cannot signify that no glucose is being utilized, or that sugar which is excreted represents either the partial or complete amount which is being formed from protein alone.

Shorr, Loebel and Richardson (138) present experiments with both living rats and excised rat tissue. Excised renal tissue from normal rats and from phlorhizinized rats responded to the presence of glucose by an increase in oxygen consumption and corresponding rise in the R.Q. The average R.Q. 0.850 indicated that carbohydrate was furnishing about 50 per cent of the energy. This work is against the theory that phlorhizin interferes directly with the oxidation of carbohydrate by the cells in general. Bach (6) reported that carbohydrate synthesis in liver slices is partly suppressed by sodium fluoride and iodoacetate, but is apparently increased in the presence of phlorhizin.

Fleischmann (44), however, from the study of excised tissues believed that phlorhizin blocked the mechanism for sugar absorption in the body as well as the tubular epithelium of the kidney. Wetzel and Zitzewitz (157) report a rise in the R.Q. of slices of liver and diaphragm put in Ringer's solution containing 0.2 per cent glucose. This phenomenon is completely inhibited by phlorhizin.

The consensus of opinion is that there is no essential defect in the utilization of sugar caused by phlorhizin but the lack of utilization is due to its very rapid loss through the kidney.

Intermediary metabolism of protein. Loewi (85) first showed that amino acids originating from the proteolysis of meat could maintain nitrogen balance

even as protein itself. Stiles and Lusk (143) fed a dog a meat pulp which had stood 14 months with trypsin proteolysis and concluded that prolonged digestion destroyed one of the principal dextrose producing substances, so that only a 40 per cent conversion of the ingested materials into dextrose resulted.

It has been shown by various workers (90, 125) that glycocol and alanine are completely converted to dextrose, and that three of the four carbon atoms of aspartic acid and three of the five carbons of glutamic acid are changed to dextrose. Tyrosin had no influence on sugar excretion. Propionic acid (124) is completely converted to glucose, and methionine (146) is also a sugar former.

Dakin (29) in 1913 summarized the data relating to glucogenetic properties of the amino acids as recognized at that time. Serine, cystine, proline, ornithine and arginine are all capable of yielding large amounts of sugar when given to glucosuric dogs. Valine, leucine, isoleucine, lysine, histidine, phenylalanine and tryptophane yield little or no sugar. He concluded that amino acids derived from protein which may yield glucose freely are all those containing two, three, four and five carbon atoms with the exception of valine. Arginine is the only amino acid with more than five carbons which may furnish glucose freely. All straight chain amino acids yield sugar except lysine, while the amino acids with branched chains, including valine, leucine and isoleucine, furnish little or possibly no sugar. Proline is the only cyclic amino acid yielding much glucose. None of the aromatic amino acids yields glucose in considerable amount.

Corley (24, 25) reported that B-alanine and ϵ-amino caproic acids are not sugar formers but γ-hydroxybutyric acid is a sugar former in the phlorhizinized animal. Hydracylic acid, δ-hydroxyvaleric acid and ϵ-hydroxycaproic acid produced no sugar. He believed these results indicated that successive stages in the breakdown of the diamino acids may result in the acids with one less carbon atom, with an amino group in the ω-position, the corresponding ω-hydroxy acids, and the corresponding dicarboxylic acids.

Janney (64) discovered that each protein produces a definite amount of glucose in the phlorhizinized organism. The various yields represent 50 to 80 per cent by weight of the protein fed. These yields approximate the ratios which the glycogenetic amino acids of the protein in each case bear to the total amino acids as determined by hydrolysis.

The extra glucose in phlorhizinization is derived from the protein fed rather than from the fats, glucose or protein of the body (61). This is indicated by the fact that glucose fed to phlorhizinized dogs in the same amounts as the extra sugar excreted after feeding glucogenetic substances reappears quantitatively in the urine. Administration of substances incapable in view of their chemical structure of being converted into glucose has been found not to be followed by extra glucose formation. In case of ingested proteins, the extra glucose elimination rises and falls nearly parallel with an extra nitrogen elimination. It is evident that dextrose must be considered one of the chief intermediary products of protein metabolism.

Mirsky, Heiman and Swadesh (103) report on the nitrogen sparing action of glucose. The rate of nitrogen metabolism is greater than that of the normal in

both phlorhizinized and completely depancreatized dogs, and intravenous glucose in large amounts causes lowering of this high rate of protein metabolism.

Gray, Ivy and Cuthbert (54) fed casein to phlorhizinized dogs and depancreatized dogs and came to the conclusion that such animals give low glucose yields from ingested protein and suggested that there was impairment of gluconeogenesis. They confirm the significant difference between the D:N ratio obtained during fasting experiments on depancreatized and phlorhizinized dogs.

Janney (64) confirmed the earlier findings that the glucose urinary excretion curve reaches its peak before the nitrogen curve. It is possible that the greater permeability of the phlorhizin poisoned kidney for glucose may explain this phenomenon. Csonka (27) found that the rapidity of absorption and elimination of ingested glucose by the phlorhizinized dog is almost the same as the rapidity of the absorption of isoglucogenic quantities of glycocoll or alanine and of the elimination of the synthetic sugar produced from them after deamination. The amount of extra sugar elimination in phlorhizin glucosuria appears to be a better index of the intensity of the hourly metabolism of the amino acids than does nitrogen output under these conditions.

Lusk (90) noted that protein catabolism in phlorhizin glucosuria is increased very greatly above the starvation requirement. Ringer (123) analyzed the whole protein metabolism into three components: 1, minimal nitrogen requirements; 2, dextrose nitrogen; 3, complementary nitrogen. Nitrogen of no. 1 can be replaced only by protein; nitrogen of no. 2 can be spared by a sufficient supply of carbohydrates (50 per cent of the caloric requirements). It cannot be spared by fat. Nitrogen of no. 3 can be spared by any foodstuff.

Experiments support Landergren's theory "that in starvation a certain fraction of protein is metabolized for the formation of glucose, and this fraction can be spared by carbohydrates and fat."

If protein metabolism is dependent to a certain extent on the concentration of glucose in the blood and body fluids, then the feeding of glucose to phlorhizinized dogs in proper quantities ought to cause a lowering in protein metabolism, while in depancreatized animals the sugar should have no effect on it. Ringer concluded that in phlorhizin glucosuria the protein metabolism rises in part because of the hypoglycemia and that the giving of glucose did spare protein even though all the glucose given was eliminated and none of it was burned.

Chambers and Lusk (17) found the S.D.A. of glycine as measured by its calorigenic action, to be dependent on the amount of glycine administered and to be independent of the size of the animal which received it.

Lusk (89) reported that the influence of work, sufficient to double the fat metabolism, has no effect on the D:N ratio. A rise in protein metabolism, as indicated by the urinary nitrogen findings, accompanied the mechanical work only when extra carbohydrate was also eliminated.

Soskin (141) in a review of the blood sugar and factors affecting it, states that there is no factual basis for concluding that phlorhizin alters the bio-chemical processes in such a manner as to allow a larger proportion of the protein molecule to be converted into sugar. And, if a constant proportion of the protein molecule is convertible, then either the depancreatized animal always utilizes a

significant fraction of the sugar derived from protein, or the phlorhizinized animal must be forming sugar from fat as well as protein.

Biasotti and Houssay (11) (s.v. p. 262) noted that in hypophysectomized-phlorhizinized dogs on a meat diet, the loss of weight and glucosuria are much less than in normal controls. They believed that the capacity to form sugar at the expense of the endogenous protein was thus greatly diminished by hypophysectomy.

Howland and Hawkins (63) fed plasma protein by mouth to fasting phlorhizinized dogs and noted that it was ingested with partial conversion to sugar. When injected intravenously, the protein disappeared promptly from the blood stream. No protein was lost and no excess elimination of sugar and nitrogen in the urine occurred, and nitrogen conservation was apparent in the after period. They postulated that the animal did not have to break the protein down to amino acids before utilization of it but rather that the protein was partially catabolized to larger protein aggregates and these reassembled to form the desired type of tissue protein. Hawkins and his co-workers, unpublished data, administered a mixture of four sugar forming amino acids by mouth and by vein to glucoside treated dogs. This mixture was partially broken down with increase in sugar and nitrogen excreted in the urine. As would be expected, the extra sugar and nitrogen recovered was the same whether the mixture was fed or given parenterally. When casein digest was given by mouth, it was partially converted to sugar and nitrogen with resulting increase of these substances in the urine. A similar reaction occurred when it was given intravenously, but more conservation was noted in the after period. This suggests that the digest when given parenterally is more effectively utilized under the conditions of the experiment. These data confirm the value of casein digests as an intravenous food and potential tissue repairing and replacement agent.

Intermediary metabolism of fat. It is long since a matter of experimental demonstration that fat may be formed from carbohydrate and hence from protein. It is believed that carbohydrate does not form from fat. Fat feeding even in excess of the total energy requirements is without effect on the D:N ratio of phlorhizinized animals. Work capable of more than doubling the fat metabolism has no effect on the sugar output of the completely diabetic organism. These facts indicate according to Nash (110) that there is no doubt that fat cannot be converted to sugar.

Lusk (89) found that phlorhizinized fasted dogs exposed to cold and mechanical work showed great increase in their fat metabolism, but there was no increased sugar production as the result of the fat combustion.

Hawley (56) studied dogs with fatty livers or fat-fed dogs and could find no evidence of gluconeogenesis even when conditions were such that the animal needed carbohydrate. These dogs were given insulin in addition to the phlorhizin. Page and Young (115) injected phosphotide, 100 cc. of a 10 per cent aqueous brain emulsion, intravenously and found no appreciable alteration in the urinary D:N ratio in glucoside treated dogs. This is further evidence against the conversion of fat into sugar.

Mineral metabolism. The effect of phlorhizin on the mineral metabolism of

the body is a comparatively recent subject and was studied originally as a result of its close relationship with some of the more obvious phenomena of phlorhizin glucosuria. Nash (108) noted, in close parallel to the repression of the ketosis of fasting phlorhizinized dogs following glucose ingestion, there is a sharp drop in the inorganic phosphorus of the blood. Coincidentally, the inorganic phosphorus of the urine diminishes rapidly and most frequently disappears entirely. A definite renal threshold for phosphate is indicated. The larger the dosage of sugar, the more pronounced and prolonged the effect on the urine phosphates. During this period of heavy sugar excretion, the urine reaction changes from strongly acid to faintly acid or neutral. Harrop and Benedict (quoted by Nash, 110) suggest that the phosphate withdrawn into the tissues, as noted in normal animals, is concerned with an intermediary stage in the storage of carbohydrate as glycogen, which occurs in phlorhizinized animals. It may be that the "alkaline tide" after meals is a reflection of such a process. The prompt recovery of the alkaline reserve in the phlorhizinized dog after dextrose feeding may not be due entirely to the burning of ketone bodies. It is possible that the elevation of the carbon dioxide combining power of the blood in human diabetics after insulin administration likewise may be largely attributable to glycogen synthesis and the withdrawal of blood phosphates. Henderson (57) presents the idea that the condition chiefly determining the extent of this withdrawal of alkali, or its return to the blood, is the freedom with which sugar is oxidized in the tissues.

Phlorhizin given for 7 days to dogs on a constant diet of lean beef by Kastler (67) produced a depression of potassium and an elevation of serum inorganic phosphorus in the blood. Sodium, calcium, magnesium and chlorine in the whole blood remained unchanged, as did the NPN. In spite of continued weight loss by the animals, the potassium and phosphorus values returned to normal in the after period. There was no compensating change in the potassium or phosphorus in the urine during either the phlorhizinization or the after periods that was not accounted for by slightly increased protein catabolism.

Lambrechts (78) found that phlorhizin raises the threshold of the renal excretion of phosphorus, while it lowers that of glucose.

Yamada (168) reported the total phosphorus of the blood to be decreased markedly by epinephrine, phlorhizin and glucose administration.

Ziegler and McQuarrie (169) found effects of phlorhizin alone on the blood constituents similar to those of Kastler (67), except for the absence of a significant increase in the serum inorganic phosphorus. While the serum potassium was regularly reduced by phlorhizin alone, it remained unchanged when phlorhizin administration was accompanied by the ingestion of potassium chloride in large amounts. On the other hand, the reduction of serum potassium as a result of phlorhizin administration was apparently accentuated by the feeding of excessive amounts of sodium chloride. In neither instance, however, did the extra salt ingested significantly influence the degree of phlorhizin hypoglycemia. The glucosuria due to phlorhizin administration was likewise essentially unaffected by either salt. The slight increase observed in some experiments was obviously due to accentuation of the phlorhizin diuresis by the salt ingested.

Slight variations, which were noted in the excretion of nitrogen and of minerals, could be accounted for in the same way. The calcium, magnesium, sodium, NPN, chlorides and carbon dioxide content of the serum were not significantly altered by the procedures employed. The conclusions from these experiments are that the carbohydrate metabolism due to phlorhizin poisoning bears no relationship to that of diabetes mellitus as regards its response to ingestion of excessive amounts of sodium and potassium salts. The characteristic effects of these salts on hepatic glycogenesis in the normal dog appear to be abolished by phlorhizin.

McQuarrie, Thompson and Anderson (96) found that feeding large amounts of sodium chloride increased the ability of diabetic children to utilize carbohydrate as shown by the marked decrease in fasting blood sugar and glucosuria. Potassium salts had a diametrically opposite effect, one part potassium counteracting the effect of three chemically equivalent parts of sodium.

Phloretin. It is generally observed that phloretin, which, with phlorose (glucose) constitutes the products of the acid hydrolysis of phlorhizin, does not act as strongly as phlorhizin. Ringer (quoted by Lusk, 90) gave a fasting dog 10 grams of phloretin by mouth without producing a glucosuria, and found a remarkable glucosuria only when the phloretin was given with meat. This indicates that digestive activity is necessary for the resorption of phloretin.

Lusk (90) gave phloretin and dextrose subcutaneously at the same time to rabbits without producing a glucosuria. From this he inferred that the subcutaneous tissue does not have the ability to reconstruct phlorhizin from its two components.

Lambrechts (76) prepared synthetic phloretin to test its glucosuria producing properties. He verified the purity of his product by his spectrographic method (72) (s.v. p. 256). He used several other phlorhizin derivatives, namely, phloretine, phlorglucine, phloretin prepared by hydrolysis, phlorglucine mono-ethyl ether, heptacetate of phlorhizin, and trimethyl phlorhizin. He concluded that glucose such as is present in phlorhizin is dispensable because phloretin itself is relatively active. On the other hand, trimethyl phlorhizin is totally inactive in spite of the presence of glucose. He thought that the esterification of the phenolic (OH) group may be the cause of inactivity (heptacetate of phlorhizin becomes active on saponification, while trimethyl phlorhizin becomes completely inactive). Independent of these facts, Lambrechts observed a glucosuria of sufficient importance (40 grams per liter) without polyuria. The final conclusions are that glucose is not indispensable in the structure of the phlorhizin derivative producing a glucosuria. The OH groups are very important for their blockage suppresses activity. Glucosuria without polyuria is observed.

Bach (6) however, observed that phloretin, unlike phlorhizin, partly inhibits both oxygen uptake and carbohydrate synthesis in liver slices if present in a concentration higher than 0.001M. But when glucose is added simultaneously, phloretin showed an effect similar to that of phlorhizin on carbohydrate synthesis. This indicates that the effect of phlorhizin is probably due to the complete glucoside rather than to its constituents.

Influence of drugs and poisons on phlorhizin activity. If one gives phorphorus

to a fasting animal, the metabolism is disturbed in that the total nitrogen and ammonia of the urine increase, lactic and amino acids accumulate in the organism and are excreted in the urine (90).

Ray, McDermott and Lusk (120) found that phosphorus poisoning, in dogs made glucosuric with phlorhizin, does not materially change the protein metabolism or the sugar excretion. The following explanation is offered. In phlorhizin glucosuria the high protein decomposition is due to the non-combustion of the carbohydrate radicle of the protein molecule. In phosphorus poisoning the high protein decomposition is due to the conversion of this carbohydrate radicle into leucine, tyrosine and fat (fatty degeneration). Wherever the sugar from protein is not burned, there are found pathologically hungry cells, which attract fat to themselves in larger quantities than can be utilized (fatty infiltration). Infiltration takes place in the tissues in phlorhizin glucosuria. Both infiltration and degeneration take place in phosphorus poisoning, principally in the liver.

Lusk (90) found that pilocarpine increased neither the sugar nor the phosphate secretion in phlorhizin glucosuria. In this respect the secretory cells of the kidney were thought to be dissimilar from other secretory cells.

According to Anderson and Anderson (2, 3), atropine and ergotamine are found to have no definite effect on the D:N ratio in fully phlorhizinized rats on a protein-fat diet. Nor is there any evidence obtained of any action of pituitrin on phlorhizin glucosuria in rats. Ergotoxin was found to cause a rise in the D:N ratio and the percentage of sugar in the urine of completely phlorhizinized rats on a protein-fat diet.

Clinical considerations of phlorhizin glucosuria. Much work has been done to try to establish a "phlorhizin test" as a reliable indicator of kidney function. Work on dogs (page 260) showed that in far advanced kidney disease, no sugar appears in the urine after phlorhizin injection.

Delmare (as reported by Lusk, 90) published a monograph on his studies on human beings. He established the reaction to phlorhizin in normal and abnormal cases and noted alteration in the degree and rapidity of glucosuria in the ones with disturbed kidneys. Casper and Richter (16) separated the urine of the kidneys by cystoscopy. Normal kidneys secrete sugar at the same rate after phlorhizin injection, but a pathological kidney showed a marked decrease in sugar excretion over its healthy sister organ. In cases of neoplasms, pyonephrosis and nephritis, the sugar excretion becomes minimal or disappears altogether. They were convinced that the test was of much value but Lusk (90) concludes, after study of all the evidence, that "it is well to assume that the phlorhizin test in kidney insufficiency possesses little diagnostic value."

Benedict and Lewis (9) attempted to make clinical application of phlorhizin to the treatment of malignant tumors. They reported retrogression of rat tumors after phlorhizinization, but Wood and McLean (164) were quick to criticize this report as the "Buffalo rat sarcoma" undergoes spontaneous absorption in 40 per cent of the cases.

Schaller (133) used phlorhizin in an attempt to show that there is no ante-

partum urine secretion by the fetus. The fetal kidney is affected by phlorhizin as in newborns he found traces of sugar in the urine if the glucoside was given within 48 hours of delivery. He reported finding no sugar in the amniotic fluid and concluded that the fetal kidney excretes no urine.

Hamnitzer and Joseph (Nash, 110) claim that 2.5 mgm. of phlorhizin injected subcutaneously regularly produces in pregnant women a significant glucosuria in one-half hour. In 80 control experiments, all but 7 women reacted negatively. This pregnancy test has not been accepted.

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THE PHYSIOLOGY OF THE PROSTATE GLAND

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From the research standpoint, the prostate gland is perhaps of greatest interest for 3 reasons: its dependence on hormones, the remarkable nature of its secretion, and the frequency of neoplastic growths in the gland in several species including our own.

The female prostate. Para-urethral glands homologous to the male prostate and with identical histological appearance occur in certain female creatures. In women Virchow (139) observed small nodules in the proximal portion of the urethra, which sometimes contained concretions similar to the prostatic calculi of men. Most of the observations concerning the para-urethral glands and the effects of hormones upon them have been made in the rat. The glands are located caudal to the bladder in a position similar to the ventral prostate of the male; they are pale pink in color, contain a watery secretion, and the weight may reach 70 mgm. (47). The incidence of prostate glands in females is affected by genetic influences; in some strains of rats (92) the incidence was 93 per cent while in other strains no vestiges or rudiments were discernible. In the gross the prostate of females is visible normally only in early life and in the last third of pregnancy, as well as in the first 2 weeks of subsequent lactation (10); exception—in the multimammate mouse, *Mastomys*, a large prostate is present in all normal females (5) and it hypertrophies during the luteal phase of estrus. In 1 strain (92) primordia of the prostate were first observed in both males and females at $19\frac{1}{3}$ days of fetal life; 10 days after delivery the ducts and lobules showed lumen formation and on the 25th day most of the acinar cells showed evidence of secretion, while between age 30 and 40 days evidence of involution occurred (92, 115); the gland persisted for the whole lifetime of the rat but continued to show this involuted appearance (80).

The female prostate is stimulated by androgens and depressed by estrogens and castration. Korenchevsky and Dennison (80) found a greater incidence of prostates with larger size in females injected with androgen than in uninjected controls and testosterone caused the formation of the prostate in ovariectomized rats (19, 78); dorsolateral prostates and coagulating glands can be induced in females with sufficiently large dosages of this hormone (93). In a series (47) of normal untreated virgin rats the incidence of prostate was 9.4 per cent, in rats injected with estrone 0 per cent, and in rats injected with testosterone propionate 58.3 per cent. The glands are atrophic in spayed females (80). Price (117) transplanted female prostatic tissue into other young and old, castrate and intact rats; the development was mostly good in normal males and castrate females and was poor or absent in castrate males and normal females.

The rest of this paper is concerned with the prostate gland of the male.

Essentiality of the prostate. Clearly the prostate is unessential to mammalian life since in some females it is absent. It is also unessential for fertility; Young

(150) was able artifically to inseminate guinea pigs with sperm from the isolated epididymis, with resulting pregnancy. Excision of the prostate and seminal vesicles abolishes fertility (131). No doubt the prostate is essential for fertilization by the natural method in mammals since the amount of fluid in which the sperms are contained in the vas deferens is so small that, without dilution, it could hardly be delivered from the urethra at ejaculation; thinning and increasing the volume of the sperm, then, are functions of the prostate.

The prostate is not an endocrine gland. It has been claimed that the prostate gland elaborates an internal secretion, but the evidence is oblique and unconvincing; the arguments are based on 3 types of experiments. It was stated (128) that prostatectomy in dogs was followed by cessation of spermatogenesis and that the feeding or injection of glycerin extracts of the prostate prevented the atrophy; however the caging of dogs itself (64) is often followed by atrophy of germinal cells which recover spontaneously, the housing and diet remaining unchanged.

Extracts of the prostate of the bull, both lipid and watery, injected into rabbits for many days (77, 81) sometimes produced a negative nitrogen balance; the increase of nitrogen excretion was greater when simultaneous extracts of prostate and testis were injected. The results were inconstant. It was suggested (77) that the prostate has an internal secretion affecting nitrogen metabolism and that it is in a synergetic relation with the testis.

Macht (87) found that desiccated prostate fed to tadpoles hastened somewhat their metamorphosis into frogs, although not so rapidly as feeding thyroid substance; in addition to these slight effects there occurs greater shortening and differentiation of the alimentary canal (53) than in the control tadpoles. The effects of prostatic substance seem not to be specific (118) in promoting mettamorphosis since extracts of many organs had comparable effects. Prostate feeding (89) had no effect on behaviour or ability to solve maze problems in rats.

Investigative techniques. The principal methods of investigation of prostatic function depend on determination of size of the gland, its cytology and quantitative and qualitative studies of its secretion. The cytological evidence of secretion consists of light areas in the lumen region of the cell which appear in the rat at age 12 days (114) and disappear in the adult within 4 days after castration (101); clearly this morphologic type of evidence is largely qualitative and inferential.

Prostatic transplants grow well in the anterior chamber of the eye (51, 83, 107) and variations in size have been recorded by photography. The grafts decrease in size after orchiectomy and increase when androgen (104) or gonadotrophic hormones are injected (83, 104); continued injections of a single type of gonadotrophin (e.g., chorionic) however do not lead to long continued progressive increase of size (83, 104), apparently because of immunity to the injected protein since later administration of another type of gonadotrophin, such as pregnant mare serum, causes an additional response.

Study of the secretion has provided quantitative data indicating more delicate changes in the prostate than the foregoing methods. The secretion may

be studied directly or by indirect methods. Indirect methods concern measurement of acid phosphatase in the blood or urine; acid phosphatase values in serum are increased in certain patients with metastatic cancer of the prostate (45) and serial study of this enzyme in clinical patients gave the first indication of the activity of hormones on disseminated human prostatic cancer (62). The urine of normal adult men (125) contains 3- to 5-fold greater amounts of acid phosphatase than that of women or children and, remarkably, the daily amount of enzyme excreted in a single individual is nearly constant.

Direct studies of prostatic fluid have been made principally in man by massage of the prostate and collection of the secretion from the urethral meatus; surgical procedures have been used in the dog and the boar (96).

Eckhard (24) first prepared a fistula of the prostate in the dog and discovered that stimulation of the *nervus erigens* caused abundant prostatic secretion; the dog has a well developed prostate but no seminal vesicles. Farrell (31) devised an operative method in 2 stages for the dog whereby many acute observations were made. At the first operation the bladder is separated from the prostate and an anastomosis is made between the bladder and penile sheath, followed later by establishing a urethral fistula in the perineum. Difficulties in the collection of fluid made chronic observations impossible in this otherwise ingenious technique. The first quantitative collections of prostatic fluid in the dog for long periods involved a new technique (64) whereby the urine was deviated through a metallic cannula after isolation of the prostate from the bladder and a ventral slitting of the prepuce; the dogs survive for long periods in good condition and collection of prostatic fluid is easy and complete.

The secretion of prostatic fluid. The prostate excretes small amounts of fluid at frequent intervals (resting secretion) and the secretion is greatly augmented by parasympathetic stimulants (stimulated secretion); resting secretion is discharged usually from the urethra in the urine. Judging from the daily amount of acid phosphatase excreted in the urine (125), average 613 units per day, the amount of prostatic fluid secreted by man is in the order of 0.5 to 2 cc. per day. In the dog resting secretion is about 0.1 to 2 cc. per hour (3, 34); after the urine has been deviated by the prostatic isolation operation (64) the "tree reflex" is well preserved and dogs approach the legs of laboratory tables and void prostatic fluid at frequent intervals.

Prostatic secretion is primarily a function of androgenic activity, and secondarily of chemical and nervous stimulation; rises of acid phosphatase in the urine occur after sexual stimulation (125) in man. In dogs frequently a steady output occurs for months while less commonly a plateau of secretory activity is interrupted by high peaks (64). Normal dogs may secrete as much as 4 times the weight of the prostate in 1 hour, but very large glands in spontaneous cystic hyperplasia do not give correspondingly large amounts of secretion, although the cysts communicate with the ducts; for example, a dog (60) whose hypertrophied prostate weighed 102 grams secreted from 7 to 11 cc. whereas a young normal adult of the same body weight secreted 24 to 38 cc., the prostate weighing 10.8 grams.

Pilocarpine hydrochloride, 6 mgm. intramuscularly, provides a good output

of secretion in normal dogs, usually without accompanying emesis and about 95 per cent of the secretion occurs in the first hour after injection. Increasing the amount of pilocarpine increases the secretion (64) but not in a simple linear relationship and the gland does not become refractory to pilocarpine, although some signs of "prostatic fatigue" are seen after repeated stimulations in a very short time.

It was claimed (33) that occlusion of the outflow of prostatic secretion caused atrophy of the gland but the experimental technique was defective; plugging the ducts with a transplant of muscle (61) did not lead to prostatic atrophy in dogs injected with androgen.

Starvation of dogs injected with androgen for 3 weeks led to an increased secretion of prostatic fluid and definite growth of the prostate; Pazos (109) observed further that the composition of the secretion was not disturbed by inanition.

Effect of nerve stimulation and drugs on prostatic secretion. There is no secretion from immature (99) or castrate dogs (64). The secretory pressure of the adult canine prostate has been found to be 16 to 18 (99), and 17 to 26 (34) mm. of mercury respectively. Stimulation of the hypogastric nerves (34, 99, 127) and the *nervi erigentes* (24, 99, 148) induces prostatic secretion. Faradic stimulation of the hypogastric nerves produced either large (99, 126) or small secretion (148) in the dog. In man stimulation of this nerve causes ejaculation of semen (6).

Parasympathetic stimulating drugs greatly augment prostatic secretion. Epinephrin, nicotine and acetyl choline (34) caused a slight increase of secretion and pilocarpine a great increase (34, 64, 99, 148); atropine antagonizes pilocarpine stimulation and results in cessation of secretion (34, 148).

Strips of the normal prostate gland exhibit no rhythmic contractions (88, 94, 95, 140) but these occur in the prostate of castrate rats (94, 95) and the administration of androgen to the castrate reduced the spontaneous contractions. Epinephrin and barium chloride (88, 140) produced an increase of tone in prostatic strips while in most species pilocarpine added to the testing bath was without effect; in the rabbit however pilocarpine (140) produced increases of tone and rhythmic activity not detected in other animals except in the prostate of castrate rats (94, 95).

Pharmacodynamic effects of prostatic secretion. The intravenous injection of semen is always followed by a rapid fall of blood pressure (38). Prostatic fluid and extracts of the prostate gland have powerful biodynamic actions. Prostatic extracts of the dog and bull when injected in the rabbit cause a pressor effect (4, 135) followed by a marked lowering of blood pressure (26, 28), intravascular clotting and death (16, 73, 135). The pressor effect is probably due to epinephrin since this substance has been demonstrated in the prostate in considerable amounts (18, 27). Euler has studied the vaso-depressor substance, *prostaglandin*, occurring in extracts of the prostate and in prostatic fluid obtained at autopsy from man; the substance has acid properties, is dialyzable and soluble in water and alcohol; it lowers the blood pressure and increases intestinal motility in the atropinized rabbit and dilates the vessels of the hind limbs of the frog (26,

28, 29); larger quantities were found in the prostate of the adult than in children (28). Another highly active substance, *vesiglandin*, was found in the prostate of the monkey (28); this lowered the blood pressure but had no, or only a weak, effect on isolated intestine. These agents which produce vasodilatation and strong contraction of smooth muscle occur in the prostate and seminal vesicle in larger amounts than in many other organs and are assumed by Euler (28) to function in the emptying of these glands. The prostate of the sheep contains the same amounts of vasodepressor and smooth muscle relaxing substance as human

TABLE 1

Chemical composition of human and canine prostatic fluids

	MAN RESTING FLUID	DOG PILOCARPINE STIMULATION
pH	6.3–6.45 (67)	5.29–6.16 (64)
Values per liter of fluid		
Water, gram	927–936 (67)	981 ±3 (64)
Sodium, m.eq	149–158 (67)	159 ±2.6 (64)
Potassium, m.eq	28.7–61.4 (67)	5.1 ±0.2 (64)
Calcium, m.eq	28.7–32.7 (67)	0.3 (64)
Chloride, m.eq	34.8–46.1 (63)	160 ±2.7 (64)
Acid-soluble P, m.eq	0.65–1.77 (63)	Trace (64)
Carbon dioxide, m.M	3.1–5.4 (67)	0.8–0.9 (64)
Values per 100 cc. of fluid		
Total nitrogen, mgm	295–511 (67)	154 (64)
Non-protein nitrogen, mgm	30–90 (67)	22 (64)
Total protein, gram	2.46–2.64 (67)	0.8 (64)
Glucose, mgm	Trace–16.4 (63)	0–30 (64)
Ascorbic acid, mgm	0.54 (3)	0.76 (3)
Citric acid, grams	0.48–2.68 (66); 7.0 (123)	0.0026 (66)
Acid phosphatase, King and Armstrong units (76)	255–1727	3–286 (3)
Alkaline phosphatase, King and Armstrong units (76)		0–106 (3)
Total lipids, mgm	286 (124)	
Cholestrol, mgm	62–105 (124)	
	86–618	130–210 (106)
Specific gravity	1.022 (106)	1.005–1.008 (3)

prostate; the content of organs such as liver, testis and muscle was less than 1 per cent of the prostate (30). The lowered blood pressure following intravenous injection is abolished by exclusion of the liver from the circulation (29).

THE COMPOSITION OF PROSTATIC SECRETION. The chemical composition of prostatic fluid (table 1) is remarkable from the standpoints of inorganic, organic and enzyme chemistry. All of the data for human prostatic fluid relate to resting secretion obtained following prostatic massage; amounts of fluid obtained are usually less than 1 cc. so that complete analyses cannot be made on a single sample and it occasionally is contaminated with fluid from the seminal vesicle or ejaculatory ducts. Quantitative data for dog prostatic fluid are more exact since

technical difficulties are less. Compared with stimulated secretion, the resting secretion of dogs (3) is more turbid, more alkaline and contains less chloride; observed values, pH 6.6, chloride 104 m. eq. per liter.

Inorganic. The prostatic secretion is more acid than plasma. Stimulated dog prostatic fluid approximates 0.16 normal NaCl (64). Human prostatic fluid contains considerably larger amounts of sodium, potassium and calcium (67) than plasma; on the other hand there are deficient anions with low levels of chloride, bicarbonate and phosphate. Other anions, namely, the amino acids (20 mgm. per cent), proteins—peptones and proteoses, and citrate balance the cations inadequately for the observed pH of 6.45.

Organic. Most of the proteins of human prostatic fluid are derived proteins which are not coagulated by heat and pass readily through semi-permeable membranes—proteoses (39, 67, 111, 119). The content of glucose in prostatic fluid is exceedingly low (63). Scherstén (123) and others (21, 66) found that human prostatic fluid is rich in citrate. No doubt citric acid is a factor in the accumulation of calcium, since other fluids rich in calcium salts (e.g., milk and urine) are rich in citrate, and citrate also functions in preventing the precipitation of calcium salts; however precipitations of calcium phosphate (apatite) and cholesterol in the form of calculi (59) often occur. Even in young men such apatite deposition occurs in the "corpora amylacea." The spermine content of human prostate is remarkably high (49) (0.1 gram per 100 grams of fresh tissue) and probably most of the spermine of semen is derived from this organ (48); this base does not activate spermatozoa.

Enzymes. a. *Phosphatase.* Kutscher and Wolbergs (85) discovered that acid phosphatase is present in prostate gland and prostatic fluid in very large amount. This enzyme is a chemical secondary sex characteristic; Gutman and Gutman (42) observed small amounts in infancy increasing during puberty to high values in the adult and that traces in the prostate of immature monkeys (43) became greatly augmented by androgen administration. No function for this remarkable enzyme has been discovered.

b. *Fibrinolysin.* Proteolytic enzymes were discovered in prostatic fluid (66) in a study of liquefaction of human semen when it was found that these fluids rapidly liquefied clotted blood and fibrin. Human semen contains fibrinolysin derived in large amount from the prostate gland. This enzyme, a physiological constituent of the prostate of man, resembles closely streptococcal fibrinolysin (137) previously recognized only in association with bacteria. Human blood is lyzed well by this enzyme, which lyzes rabbit blood poorly and beef blood not at all. Contrasted with trypsin, human prostatic fluid does not act on denatured hemoglobin (66) and digests fibrinogen with difficulty; contrasted with streptococcal fibrinolysin, the blood of men resistant to this enzyme is easily lyzed by prostatic fibrinolysin—an immunologic difference; both streptococcal (97) and prostatic (98) fibrinolysins are inhibited by crystalline trypsin inhibitor.

c. *Fibrinogenase.* It was observed that the prostatic fluid of the dog destroyed fibrinogen more readily than fibrin, the activity being attributed to fibrinogenase (66). Both fibrinolysin and fibrinogenase are chemical activities as yet unidenti-

fied as chemical entities. Both human and dog prostatic fluids contain these activities but in different concentration. Human semen contains much fibrinolysin, little fibrinogenase; dog semen contains little fibrinolysin, much fibrinogenase. The fibrinogen of man in certain disease states is not destroyed by dog fibrinogenase (66). These agents may be maintained for years in refrigerated prostatic fluid.

d. *Miscellaneous.* The prostatic secretion of normal dogs (70) clots oxalated or heparinized rabbit and beef plasmas (these fluids are resistant to the activity of fibrinogenase) but does not clot fibrinogen alone; human prostatic fluid does not coagulate oxalated plasma. The tryptic activity of dog prostatic fluid ranges from 0.1 to 1.1 units. Thromboplastin (66) is present in prostatic fluid as in the juice of many other tissues.

The prostate gland and fluid of the dog have been reported to contain amylase (75). We have confirmed this observation but found it to be inconstant. A germicidal power (149) in dog prostatic fluid has been described against B. coli, S. aureus and streptococci; neither complement nor lysozyme was demonstrated. Camus and Gley (14, 15) found that the prostatic fluid of the guinea pig contains an enzyme, *vesiculase*, which coagulates the seminal vesicle secretion; it is unable to clot blood or milk. Diamino oxidase is present in considerable quantity but is less concentrated (151) in prostatic tissue than in semen.

Drugs. Alcohol (32) is excreted in dog prostatic fluid (90–594 mgm. per cent) after ethyl alcohol administration; sulfonamides (35, 58, 84) likewise are excreted.

Lipids of the prostate. Thompson (136) described the laminated corpora amylacea normally found in the prostate of adult man and monkey (and only in these species) and described yellowish refractile bodies in prostatic fluid. Fürbringer (36) discovered that the opalescence of prostatic fluid is due to fat droplets which he named "lecithin granules"; this lipid displays double refraction in polarized light (112, 113). Lipids are easily demonstrated in the epithelial cells (110, 147) and their presence in prostatic fluid represents a true secretion. The cholesterol content (133) of normal prostatic tissue (0.8 gram per cent) is lower than that of prostatic adenomas (1.10 grams).

Scott (124) found an average total lipid content of human prostatic fluid of 286 mgm. per cent, approximately half the concentration found in human plasma. Total phospholipids constituted over 60 per cent of the total lipid value. The moist-ether soluble phospholipid consisted of cephalin. Lecithin was not demonstrable indicating that the term "lecithin bodies" is not applicable to the fat bodies found in prostatic fluid. His values for total cholesterol in prostatic fluid ranged from 62 to 105 mgm. per cent, considerably lower than the range of 86 to 618 mgm. per cent reported by R. A. Moore (106). These analyses indicate that there is little if any neutral fat in prostatic fluid, the phospholipids and cholesterol accounting for almost all the total lipid found. Microscopic examination of prostatic fluid from normal men reveals lipids in gross aggregates while the lipids of plasma are of colloidal particulate size or are in solution. In certain prostatic inflammatory states much or all of the lipid is found in macrophages (colostrum corpuscles) in the prostatic secretion.

Effect of prostatic fluid on spermatozoa. The addition of prostatic fluid to sperm is said to increase their motility; the experiments have not been carefully carried out and it is impossible to exclude non-specific factors, such as dilution of thick sperm, the effects of inorganic ions and changes in gas tension as the cause of the acceleration. Saline admixed with prostatic fluid increased sperm motility and preserved vitality longer than occurred in the saline (54, 127, 131, 148) solution used, sodium chloride, 0.7 per cent, which however itself is toxic to spermatozoa (54). The addition of dog prostatic fluid to testicular spermatozoa (which are commonly motionless or nearly so) produced active motion (143); the same result was achieved with saline.

Ivanow (71, 72) reported that prostatic fluid increased the motility of sperm temporarily but did not represent the most favorable media for life of the sperm; utilization of oxygen was less rapid in prostatic fluid than in boiled prostatic secretion or saline.

TABLE 2

Chemical composition of fractions of the ejaculate

Glucose arises chiefly from seminal vesicle; the other components are excreted chiefly or exclusively by the prostate.

COMPONENT	FRACTION OF EJACULATE	
	First	Last
Citrate, mgm. per cent (123)	770	120
Calcium, mgm. per cent (123)	52	20.4
Acid phosphatase units, 1 cc. (44)	2760	300
Glucose, mgm. per cent (91)	157	377
Fibrinolysin, minutes per test (65)	120	288

Time of emptying of the prostate during ejaculation. In man it is known that the prostate discharges most of its secretion before the seminal vesicle in, roughly, a one-two relationship during ejaculation. The evidence is chemical in nature obtained on ejaculates fractionated according to time of delivery from the urethra (table 2).

Metabolism of isolated prostatic tissue. The oxygen consumption of the prostate of the normal dog (QO_2 4.3) and rabbit (QO_2 6.2) is approximately half that of kidney. Aerobic glycolysis ($Q_L^{O_2}$ 0.97) is high indicating a deficient oxidation of carbohydrate in the normal (2). The carbohydrate utilization of human prostatic adenoma is characterized by a low respiration, low pyruvate utilization, and high glycolysis, anaerobic and aerobic.

THE RELATIONSHIP OF ENDOCRINES TO THE PROSTATIC FUNCTION. In the normal prostate the acinar epithelium is composed of tall columnar cells; 16–28 μ in the guinea pig (121); 20–30 μ in the rat (114); 14.4–27.5 μ in man (69). Secretion is only possible from this tall epithelium which in turn is a function of androgen, and when androgen is withdrawn sharp decreases in height occur (69, 101).

When orchiectomy is done in dogs, a measurable decrease of secretion is observed within 24 hours and prostatic secretion ceases 7 to 23 days after castration (60, 64). Usually orchiectomy is followed by prompt cellular regression of the prostate, changes in the cytology of the rat being observed promptly (101). There is loss of the light areas within 4 days and then progressive reduction in epithelial height, nuclear size and acinous diameter, the epithelium becoming pseudostratified and the nuclei pycnotic (116). Exceptions to this rapid involution after castration were noted in benign hypertrophy in man where decreases in height of the epithelial cells were not marked at 29 days (69) but were severe at 93 days. Also, in the guinea pig (37, 121, 122) involutionary changes develop slowly after castration. Development and maintenance of well differentiated epithelium occur in the prostate of rats and mice castrated at 1 to 3 weeks of age—the Price effect (114) and is apparently associated with the secretion of androgen by the X-zone in the adrenal cortex (55, 56) since adrenalectomy (9, 57) reduces the prostatic weight; the anomaly disappears spontaneously about day 40.

Testosterone propionate, 10 mgm. daily in sesame oil, gives measurable prostatic secretion in immature dogs after 3 to 7 injections (64), and there is a steadily rising secretory curve for at least 5 months; in hypogonadism in the male androgens initiate and increase prostatic secretion but when administered to normal man testosterone had little effect on the seminal output (52). There is further evidence that androgen is less effective in intact males than in castrates; the daily administration of 0.1 to 0.5 mgm. of testosterone propionate per kilogram to rabbits with intraocular prostatic transplants (82) produced an increase of area of the graft for 2 weeks followed by a stillstand or regression; the same treatment in castrate rabbits produced a continuous increase for at least 3 months.

Burkhart (7, 8) studied the effects of androgen on the prostate by the colchicine technique; the male hormone effects an increase of mass of the prostatic epithelial cell followed by cell division. A single dose of testosterone propionate, 0.1 mgm., in a castrate rat weighing about 170 grams causes an increase of cell volume of the epithelium of the ventral prostate detectable 23 hours after injection followed by mitotic division at 35 hours; the effects of androgen are evident for $2\frac{1}{2}$ days after this single injection when the cells revert to a castrate condition.

Soon after the introduction of crystalline androgens it was found that the characteristic cytologic effects of androgen on the prostatic epithelium could be abolished under certain conditions by simultaneous administration of estrogenic hormones (50, 74, 152, 153, 154) in the mouse, rat and monkey. Also with respect to the prostatic secretion estrogen is able to nullify the effect of androgen (60); the dosage of estrogen has been determined whereby the rising curve of prostatic secretion induced by injected androgen is converted to a plateau (neutralization), and larger doses completely vitiate the secretory effects of androgen. By this type of biological "titration" (60) it was found that daily injections of diethyl stilbestrol, 0.4 mgm., neutralized testosterone propionate, 10 mgm., similarly administered (a ratio of 1:25) and larger doses of this estrogen

caused a cessation of secretion. When estrogen is administered to a castrate or immature dog, the acinar elements remain uninvolved while in the ducts, the lining of the posterior urethra and the utriculus, columnar epithelium is replaced by stratified squamous cells; when the acini are luminated (action of androgen) this epithelium also becomes squamous and stratified (60). This metaplasia is reversible by androgenic treatment (120). Involvement of the utriculus with squamous cells is the first cytologic sign in the dog of estrogen dominance.

The prostate gland in late fetal life and in new born boys usually is the site of epithelial metaplasia in the utriculus and the collecting ducts (1); this was attributed for theoretical reasons (46) to penetration of hormones from the mother through the placenta. The metaplasia is due to estrogen. Burrows (11) produced these changes by injecting estrogen in adult mice and restitution to the normal state followed a cessation of estrin; estrogen administered to infants (129) maintained these changes for 7 months—an age when the prostate in normal babes would have resumed the customary inactive appearance.

In the intact mouse (12, 86) dog and monkey (141) administration of estrogen for long periods leads to urinary retention with uremia and death of the animal; the posterior lobe of the prostate and the utriculus become enormously distended with retained secretion. In the rat (79) and monkey (153) male hormone administered with estrone completely prevented the pathological changes in the prostate. Since the introduction of anti-androgenic methods of treatment of cancer of the prostate by Huggins and Hodges (62) considerable numbers of men have been treated with large doses of estrogen for prolonged periods without "estrogenic urinary retention"; man is, apparently, the only mammal in which this phenomenon does not occur. Estrogen (138) increases growth of muscle and connective tissue of the prostate.

Sharp decreases in prostatic secretion in the dog follow within 1 day after injection of an adequate amount of estrogen which, when maintained, leads to cessation of secretion in about 10 days (60); desoxycorticosterone acetate (68) causes only a slight secretory depression. The responses of the prostate to both androgenic and estrogenic stimulation appear to be unaffected by section of the hypogastric and sacral nerves (133).

Androgen threshold of the prostate. The prostate has a lower threshold to androgens than the seminal vesicle. Working with crude extracts Moore and Gallagher (100) found that about 3 times as much androgen was required to show effects in the seminal vesicle as in the prostate. This has been confirmed with pure androgens (8, 108). In the administration of estrogen to man (65) it was found that the amount of seminal vesicle secretion in the ejaculate decreases more rapidly than the prostatic secretion; the ejaculate becomes less solid, liquefies more quickly and the concentration of glucose decreases more rapidly than fibrinolysin (65). There is a differential reaction of the prostatic lobes in the rat to androgen withdrawal. On the tenth day after castration involutionary changes were first detected cytologically in the anterior lobe (coagulating gland) although they had been noticed on the fifth day in the other prostatic lobes (101).

Two functionally different prostates. For many years it had been known that coagulation resulted when prostatic fluid of the rat was mixed with the secretion of the seminal vesicle; Walker (145) observed that only secretion from a specific region of the prostate, the anterior lobes (coagulating gland) could induce clotting, the middle and posterior lobes being inactive. The coagulating gland has a slightly different cytologic appearance from the other lobes although it has the same embryologic origin (25). This gland occurs in rat, guinea pig (145) and monkey (142); cross-coagulation is possible between the seminal vesicle of one species and the anterior prostate of another (142, 144) and the fluids do not induce coagulation of blood. Citrate and oxalate do not inhibit seminal clotting. The coagulation is due to the enzyme, vesiculase (14, 15). A coagulating gland has not been detected as yet in man.

Benign prostatic hypertrophy. This condition occurs spontaneously with frequency only in dog and man, but the disease differs in these species. Common to both is the requisite of senility. Nearly all ancient dogs (objective evidence—worn teeth and cataracts) with functioning testis (60) have this disturbance. In man it is characteristically a disease of men over 40 years of age (134); in a series of over 700 prostates, R. A. Moore (103) found the youngest man with a true spheroidal nodule in the prostate was 39 years of age and that 75 per cent of white men between 80 and 90 had histologically demonstrable prostatic nodules (102). The disease has not been experimentally produced.

In dogs the condition consists of cystic hyperplasia (40, 41, 130) of the entire prostate, does not produce urinary obstruction and recedes massively after castration and estrogen (60); following atrophy, androgen reconstitutes the disease in the cystic condition rather than in a normal state (60).

In man, in our experience, benign prostatic hypertrophy always involves only a part of the prostate, the periurethral region, in which location spheroidal nodules are invariably found in this disease; the posterior lobe of the human prostate is never involved by this adenomatous hypertrophy so that it is useful to think of this as a separate and unique part of the gland but Le Duc (23) has denied its existence as an anatomical entity. Also the prostatic epithelium is always of tall columnar type (69)—evidence of the presence of functioning androgens. The disease is rare among Chinese (17).

Differences of opinion exist as to the effect of castration on human prostatic hypertrophy; Deming (20) and others (103) were unable to find evidence that castration brought about atrophy of the hyperplastic tissue while White (146) and many other urological surgeons (13) observed great decrease in the size of the hypertrophied prostate. We (69) observed decreases in prostatic size and also in the height of the epithelium 3 months after castration in 2 men; however enucleable spheroids of muscle, connective tissue and atrophic epithelium remained. In men with prostatic hypertrophy, the urinary excretion of androgen and estrogen (22, 105) is decreased with respect to normal young men.

In sum, human prostatic hypertrophy is an involvement of the periurethral portion of the prostate by nodular growths occurring commonly in Caucasians, rarely in Chinese. The epithelium is invariably tall columnar, so that andro-

gens must be present in physiologically effective amounts, yet much evidence suggests that androgens are not produced in as large quantity in old age as in young men; despite this the prostate grows. Certainly prostatic hypertrophy is a neoplastic condition. There is no evidence that estrogen has a causative influence on prostatic hypertrophy. To best account for the facts, we suggest that a decreased threshold to androgen in the spheroidal nodules permits prostatic growth in the face of decreasing androgen production.

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CEREBRAL CONCUSSION

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The application of the word concussion to the commonly observed transient physiological effects of trauma is derived from the nature of the usual kind of causal injury, which is such as to give "shock of impact."[1] Such transient traumatic disorders are most obvious and most commonly observed in relation to nervous structure, and this review is particularly concerned with concussion of the brain. In this connection some further definition is necessary for there are several possible transient and unrelated consequences of head injury. The most obvious change in concussion is a disturbance of consciousness both immediate, consecutive, and remote, and the most satisfactory definition would appear to be that of Trotter (97) "an essentially transient state due to head injury which is of instantaneous onset, manifests widespread symptoms of purely paralytic kind, does not as such comprise any evidence of structural cerebral injury, and is always followed by amnesia for the actual moment of the accident." Thus is cerebral concussion differentiated from an injury of such levity that the subject is momentarily only dazed, from cerebral contusions which do not immediately interrupt consciousness, from certain delayed disturbances of consciousness related to syncope (Denny-Brown, 14) or resulting from increased intracranial pressure (epi- or subdural hematoma), and from disturbances of consciousness of purely psychological kind.

The practical importance of the subject is twofold. First there is lack of clear understanding of common sequelae to head injury, namely: liability to headaches and dizziness, inability to concentrate, nervousness or unwarranted fears, various degrees of change in personality (irritability, aggressiveness, euphoria), and intellectual impairment. These symptoms have often loosely been called "the post-concussion syndrome." Secondly, the effects of exposure to violent explosion particularly common in war include persistent anxiety, loss of memory, and difficulty in concentration with or without headache and dizziness similar or identical with the "post-concussion syndrome," without outward sign of direct injury to the head. It is therefore pertinent to inquire whether any of the phenomena of concussion can be associated with a persistent structural disorder, what is the relationship between "post-concussion syndrome" and concussion, and whether the blast of explosive produces concussion or some other related state. It should be made clear that there is already a considerable volume of clinical evidence (97, 84, 85, 16, 20) to show that the "post-concussion syndrome," though most frequently seen as a result of concussion, occurs after both milder and more severe injury. The extensive earlier literature was reviewed by Strauss and Stavitsky (93), Symonds (95) and Schilder (88). The whole problem is, however, still unsettled, particularly in the rela-

[1] Oxford English Dictionary.

tionship of post-traumatic personality changes to generalized damage to the brain. Before these questions can be answered it is necessary to inquire closely into the physiological disturbance underlying cerebral concussion.

Human concussion. Careful documentation of the events immediately following the blow to the head in man is rare, for it seldom happens that a careful record is kept of the state of respiration, pulse, blood pressure, reflexes, etc., in the earliest stage. The classical case is one of which a description is given by Polis (80). The following is a brief summary:

> The patient fell 10 to 12 feet on his head on pavement and was seen by Polis in 4 minutes. He was completely insensible with face of cadaveric pallor, pupils large and fixed, and no sign of muscular activity. Respiration was extremely slow (4–5 a minute) with unequal pauses. The pulse was full but slow (48) and very irregular. The irregularity in pulse and respiration lessened gradually after 20 minutes, when vascular tension diminished. Corneal and other reflex sensitivity reappeared after half an hour. The pulse was then 60, and respirations 12–14 a minute. Consciousness was recovered after 15 hours. There was some evidence of slight cerebral contusion, for a right sided convulsive attack with some fever occurred on the fourth day.

A case of brief concussion (5 min.) followed by extensive loss of memory of events preceding the injury is related by Gussenbauer (41) but no other details of the clinical state are given. An erroneous account of Gussenbauer's case, in which details of the case cited by Polis are interposed, is given by Kocher (58) and repeated by Miller (68).

Hutchinson (54), Kramer (60), Kocher (59), Bergmann (3, 4) and other surgeons confirm the initial areflexia without however giving case details. Similar changes are commonly noted after severe head injury, where the presence of skull fracture, cerebral contusion or laceration add uncertainty to the cranial pathology, but Polis' case may be taken as a classical pattern of the immediate disturbance in man.

The prominence of disorder of consciousness in the clinical definition of concussion has been a difficulty in the interpretation of animal experimentation. This disorder in human concussion is complex enough and deserves some detailed mention here. It is maximal in degree immediately after the injury and thereafter progressively lessens. If the disturbance is severe, an initial stage of coma ("absence of any psychologically understandable response to exernal stimuli or inner need," Brain Injuries Committee (6, 1941)), is usual. Within a few seconds or minutes (possibly as long as a few hours, *vide infra*) simple maldirected response to painful stimuli or loud demands, appear. This state is commonly called *semi-coma*. This is in time followed by a phase of automatic behavior, called mental "confusion," in which responses of increasing appropriateness are made, but where there is disorientation in time, place, and person. There is subsequently no memory of any of these states (post-traumatic amnesia). The improvement in the stage of confusion is usually so rapid that accurate observation of the changes are difficult. Occasionally the whole disorder is spread over many days, or even weeks, in cases without sign of complicating injury, and if as some contend (Symonds, 94; Denny-Brown, 16) such cases may be considered as severe con-

cussion, the progress of the disorder may then be followed in greater detail. Recognition of relatives, recovery of continence of bladder, and progressive lessening of restlessness then document the slow improvement in the state of awareness. The mental confusion that accompanies this stage has clear evidence of disturbance of perception, and several authors (Schilder, 87; Symonds, 94; Paterson, 78; Paterson and Zangwill, 79) have used the principles of Gestalt psychology in relation to the restriction of the field of consciousness to explain the confused behavior. Orientation in time is usually the last process to recover (70), and is usually coincident with recovery of the patient's memory of the day before. His continuous memory also begins from that date. He may subsequently have a memory for isolated "islands" of happenings in the period of confusion, usually of some dramatic circumstance, which he is unable to relate to the remainder of his memory.

Retrograde loss of memory is a very distinctive feature of concussion. The gap is usually of only a few seconds duration, so that the patient cannot remember the actual injury. The extent of retrograde defect is not proportional to the duration of post-traumatic amnesia (86) and is often only a fraction of a second in severe cases. During the period of confusion at a stage when he can answer questions relatively intelligently (but is still disoriented) the patient is found to have a long retrograde amnesia, commonly of years, and if very confused he may be under the impression that he is reliving events in the distant past. This retrograde loss progressively shrinks and commonly continues to diminish after recovery of orientation, eventually reaching the brief interval of true retrograde amnesia. It has not proved possible to recover either the final true retrograde loss or post-traumatic loss by methods of hypnosis or dissociation as in hysterical amnesias. There is good evidence in some cases that the patient can remember the events leading up to a mild injury, but not the actual impact, immediately he recovers from the brief coma, and yet later have this same memory buried in a longer period of retrograde amnesia (7, 73).

Experimental concussion. There has been no attempt to date to identify various states of awareness in experimental concussion except the recent work of Dow and his associates (22) who have found that conditioned reflexes in dogs were affected by blows to the head of less intensity and for a much longer time than was reflex activity at lower levels. At the beginning, therefore, the investigator has to decide what criterion he is to use for the identification of concussion in his experiments. A state of lack of reaction comparable to coma is readily recognized in the condition in which an animal is said to be "stunned," but amnesia in the human sense has no animal counterpart. The situation is further complicated by the necessity for anesthesia in the course of experimentation. The early investigators accordingly adopted the criterion of immediate death without any lesion of the brain visible to the naked eye as indisputable evidence of the occurrence of concussion.[2] It is however exceedingly difficult to accomplish this and the demonstration by Koch and Filene (58) that an

[2] For historical reviews of the numerous hypotheses that have been advanced the reader is referred to the papers of Gussenbauer (42), Bergmann (3, 4), Polis (81), Kocher (59), Duret (24), and Denny-Brown and Russell (15).

animal could be killed by a series of light blows without hemorrhagic lesions first showed that "concussion" in this sense was possible. Polis (81) in 1894 demonstrated the immediate rise and subsequent fall of blood pressure that followed each such blow, with final failure after the last blow. He proceeded to demonstrate that a greater rise in blood pressure and cessation of respiration could follow one severe blow, and be followed by fatal respiratory and vasomotor failure without hemorrhagic lesions being necessarily present. He accordingly considered concussion as being essentially the result of traumatic paralysis of the bulbar centers, and found that a tangential impact of a bullet on the cranial vault was a convenient method of reproducing the condition. Bergmann (3) had since 1880 emphasized the great slowing of the pulse which usually but not always accompanies concussion and makes this an objective part of the definition of the condition. Polis (81) noted the vagal slowing and also made extensive investigations of the mechanism of vasomotor changes which will be referred to later. The vascular and respiratory changes were confirmed by Miller (68), who also stunned unanesthetized rabbits for one minute and on recovery injected Trypan blue intravenously, on 3 successive days, finding no staining of the brain or meninges in animals that had undoubtedly been rendered immobile by the injury 5 days before. Jacob (56) had also investigated microscopic changes after recovery from stunning in animals, finding petechial hemorrhages in some, none in others, and not adopting any standard for concussion. Miller (68) repeated a simple experiment reported in 1877 by Witkowski (107) in which it was demonstrated that a frog with excised heart, in surviving for some minutes, could in that period momentarily be stilled by a blow on the head. After a few moments it could right itself and continue activity. The experimental use of "stunning" an animal to investigate concussion, and the demonstration that this phenomenon could occur without vascular lesions, and indeed without vascular mechanism had therefore been accomplished more than a decade ago, but in 1940 this evidence was still not generally accepted.

In a monograph published in 1901 Kocher (59) and his pupils described similar respiratory cessation and vagal slowing of the heart produced by transient severe pressure on the dura, and proposed a hypothesis of the mechanism of concussion based upon a conception of transient ischemia induced by the deformation of the skull, with subsequent vascular stasis. Kramer (60) and Félizet (8) had shown that the skull is deformed by pressure and had demonstrated a rise of internal pressure when the dried human skull was compressed. Knauer and Enderlen (57) with more refined experimentation found that a blow upon the skull could indeed cause vagal slowing of the heart, and a transitory swelling of the brain. Scott (90) measured the intracranial pressure under similar circumstances, allowing a weight to fall on the supported head, and found a peak pressure of 300 mm. Hg at the moment of impact of a blow sufficient to abolish any response to noxious stimuli for 2 to 10 minutes. He demonstrated that a momentary passive increase of intracranial pressure of the same degree and lasting about 5 seconds also abolished responses for 5 minutes. The case for transient anemia as explanation of concussion appeared to be complete.

The mechanism that had been demonstrated however was essentially that of

cerebral compression. Denny-Brown and Russell (15) reviewed the above and related studies, and pointed out that it had not been demonstrated that a generalized increase of intracranial pressure occurred in the most usual kinds of injury when the head was thrown suddenly and violently into motion, or suddenly stopped when in motion. It also remained to be explained why very brief pressure should produce such lasting disorder of nervous function. These investigators observed the effect of a blow on the unsupported head in lightly anesthetized cats and described an orderly succession of recovery of nervous function following its immediate aboliton by a certain degree of injury. They chose the recovery of the corneal reflex as a convenient standard by which to measure the duration of traumatic disturbance of cerebral function. Transitory abolition of the corneal reflex was seen to accompany total loss of reaction in an unanesthetized monkey accidentally observed. Such abolition, in the absence of any trace of hemorrhagic lesion in the brain, brain stem or cervical cord, was accepted as evidence of concussion. They stated objections to other criteria. Other reflexes could not be elicited with such regularity, although they also were abolished for a variable interval of time by a blow sufficient to efface the corneal reflex. It is open to any investigator to nominate which of these, or other changes he shall adopt as his criterion. Loss of the corneal reflex has been found to be a satisfactory index of the reaction by Gutiérriez-Mahoney (43), Groat *et al.* (38), Walker *et al.* (98), Clarke and Ward (12), and others. Gurdjian and Webster (40), using dogs anesthetized with morphine, have stated that their animals could be rendered briefly unconscious by head injury, as judged by lack of reaction to compression of the tail, without loss of corneal reflex, though in all moderate and severe grades of injury the corneal reflex was lost. Response to pressure applied to the tail may therefore be a more sensitive indicator of general reactivity. No doubt more sensitive tests, such as reaction to visual threat, could be used if the level of anesthesia permitted. It is an essential part of the thesis advanced by Denny-Brown and Russell (15) that the initial disturbance should be generalized and maximal, if varying in duration in various mechanisms. Just below the threshold for characteristic respiratory and vasomotor effects a brief lessening of response in corneal reflex and swallowing was found. Such heightening of threshold may well give the impression of absence of response to tail pressure or other tests. The response to tail pressure cannot in any case be accepted as indication of loss of "consciousness" as claimed by Gurdjian and Webster (40). It remains to be shown whether amnesia, which appears to be the only true index of the disturbance of consciousness, can be produced by lesser degress of injury than will abolish reflex function. Inactivity of a conditioned stimulus without impairment of the brain stem mechanism as found by Dow and Raaf (22) is interesting in this connection, though it is necessary to be certain that the effect was not just the distraction of attention such as would be produced by any loud sound or other sensory stimulus ("external inhibition").

The question of anesthesia is manifestly of great importance. Initial vinyl ether, followed by novocaine infiltration of operative fields, as used by Walker *et al.* (98), appears to be most satisfactory. Denny-Brown and Russell (15)

and Williams and Denny-Brown (103) used nembutal, controlled in other experiments by the use of ether alone, but the anesthesia with these had to be very light indeed, especially for electroencephalographic recording. Under deep anesthesia the rise in blood pressure with concussion is less marked and may be absent, the respiratory change is reduced to a simple pause, and all excitatory effects lessened or abolished. The use of morphine as anesthetic appears likely to produce equivocal reactions if a noxious stimulus is used as test indicator.

Acceleration concussion. Denny-Brown and Russell (15) observed that it was possible to graduate the effect produced by the intensity of trauma on the unsupported head. Between an intensity of injury sufficient to kill the animal with one blow (usually with contusion of the brain and upper cervical cord, but occasionally without lesion visible to the naked eye), and an intensity which did not cause loss of corneal reflex ("subconcussive" injury), was a wide range within which abolition of the corneal reflex for variable intervals of time could be brought about. The duration of the other phenomena varied with that of the corneal reflex. By means of a graduated pendulum they were able to measure the intensity of such injuries. It was apparent that the trauma under such circumstances had to have a critical velocity as well as energy, for however great the moving mass, at slow speeds of striking the head was simply pushed gently aside. If the head is prevented from moving by some clamp or heavy obstruction, the effects produced would be more directly related to the mass of the striking object, and the ability of the skull to withstand the crushing effect of momentum.

The two modes of injury are clearly different, but whether the difference is of practical importance will be discussed further below. Denny-Brown and Russell (15) reported that in 10 patients observed by them with head injury from crushing violence only one had immediately lost consciousness with retrograde amnesia, in spite of the production of cranial fractures and cranial nerve injuries in all.

By the use of the pendulum, striking the movable head, it was found possible to abolish the corneal reflexes without hemorrhagic lesion in cats and monkeys regularly at velocities at impact of 28 feet per second. It was necessary to have a striking piece of weight at least equal to that of the head of the animal, and since fracture of the skull is not desired in these experiments it is necessary to use wide striking area and choose a region of skull buttressed by thick bone, as in the occipital region. A threshold of 29.4 feet per second was reported by Gurdjian and Webster (40) and of 30 feet per second by White *et al.* (101). Walker *et al.* (98) in an illustration claim that "momentary concussion" was produced at a velocity of 9.6 feet per second. The physics of such an impact are, however, complex and the factors of mass and speed of blow are only of importance in relation to their effect on the head of the animal, for unless a depressed fracture is caused, it is the acceleration of the head that is the essential factor. In the absence of any satisfactory way of measuring this, the speed of applied blow is an approximation which is of value only as long as the effective mass of the striking force is greater than that of the head, and is varied to some degree by

the elasticity of the skull and cushioning of the scalp. Such factors also presumably explain the greater ease of inducing concussion by occipital blows in the sagittal plane than in other planes, though the difference between the effect on striking speeds is relatively slight.

It must be stressed that effects are induced by blows of lesser velocity, notably a brief but sharp fall of blood pressure and a start reflex or sudden flexion of all limbs with inspiratory gasp. This latter reflex can be induced by a blow to the neck or upper thorax in a lightly anesthetized animal. The corneal reflexes are not immediately lost, though near the threshold for concussion a transitory impairment is often found. If a level below 50 mm. systolic blood pressure is recorded for 10 to 20 seconds some delayed impairment of reflex responses may occur. In the decerebrate animal acceleration of respiration is prominent. Denny-Brown and Russell called these "subconcussive effects," attributable to stimulation of the vagal mechanism. They considered it the probable mechanism of the "knock-out" in boxing where the subject sags gently to the floor without retrograde amnesia and with relatively rapid recovery. Concussion does occasionally occur in boxing but the subject is then "knocked stiff," lies pale and immobile and has no memory of the incident. Williams and Denny-Brown (103) found no electroencephalographic changes in subconcussive effects in cats. Dow and his associates (22) have investigated 74 cases of human head injury, seen immediately after the accident, in which the speed of fall or speed of striking object could be computed with reasonable accuracy. They found that patients who had traumatic amnesia and abnormal electroencephalographic records had suffered impacts at an average speed greater than 24 feet per second, while those who had not lost consciousness and had normal or borderline electroencephalographic records had an average velocity of impact less than this. The limits of the exceptions to the general rule where patients who had been concussed at an estimated speed of blow as low as 16 feet per second, and others who had not been clearly concussed after an impact at 56 feet per second. The effective mass at time of impact is a more difficult matter to compute and may have accounted for some of the exceptions. It is also difficult to find a sharp limitation of electroencephalographic change due to focal injury from that due to concussion. The average speed therefore appears of greater significance than the limits encountered.

All the phenomena of such experimental "acceleration" concussion in the cat, except the electroencephalographic change, could be induced in the decerebrate or decorticated animal, then being free from the complication of anesthesia. The stilling or stunning of an active preparation is then regularly accompanied by loss of corneal reflex, by rise of blood pressure followed by deep and prolonged fall, by respiratory gasp and start with prolonged apnea in inspiration, and by absence of other reflexes (swallowing, knee jerk) for an interval. The course of recovery of each function was gradual. In both decerebrate and intact anesthetized animals it was found that a peripheral stimulus was without effect on blood pressure or respiration until late in recovery. Transient dilatation of the pupil was common as a delayed event, as was also

a delayed secondary rise of blood pressure, for which adrenalin may be responsible. Denny-Brown and Russell used these observations in making their hypothesis of the mechanism of the recorded events. They considered that the cycle of events in the respiratory and vasomotor centers was a pattern of the disturbance of neuronal function. They pointed out that all mechanisms may show evidence of both initial and continued stimulation (inspiratory gasp, rise of blood pressure, closure of lids, twitch of vibrissae) which indicated that the lower levels of function continued to be stimulated but were inaccessible to reflex stimulus during the duration of concussion. The duration of areflexia varied in each case, being shortest for respiration, longer for corneal reflex and vasomotor effects, and longer still for the pinna reflexes.

Some difficulties are encountered by this hypothesis. First that a convulsive gasping spasm regularly appeared in lightly anesthetized or decerebrate animals with a latency of 5 to 10 seconds after the impact. It had been found that a strong stimulus could break through the reflex block at an earlier stage of recovery than could weaker stimuli. The delayed motor disturbance, often coinciding with delayed onset of heart block, was regarded as the result of residual powerful afferent excitation remaining from initial direct stimulation of the 10th nerve roots by the injury, manifested at the earliest recovery of reflex block. Secondly, the rise in blood pressure commonly gave way to a steep fall, which sometimes proved fatal. This diphasic curve was explained as a stage of exhaustion of the vasomotor center after its initial discharge and before recovery of the reflex compensatory mechanisms. The presence of continued discharge of the center was demonstrated by the ability of a sudden passively induced rise of intracranial pressure to cut short the rise in blood pressure at any stage. Gurdjian and Webster (40) observed that the rise of blood pressure is prevented by spinal transection or by the use of yohimbin to inhibit adrenalin. Walker *et al.* (98) found continuation of hypertension after spinal section but do not appear to allow for the stimulating effect of such section or the stimulation by adrenalin already in progress. Lastly there remained the difficulty in accounting for the occurrence of a simple fall in blood pressure and simple cessation of respiration with loss of all reflex and motor behavior following a severe blow in deeply anesthetized animals. Here the anesthetic was considered to have impaired reflex function and reaction and the trauma to have had a purely or almost purely paralyzing effect. Hemorrhage has the same effect, as noted by Polis and ourselves. Thus it was proposed (Denny-Brown and Russell, 15) that the complicated sequence of effects of brief physical trauma could be resolved into four degress: excitation, excitation with reflex paralysis, excitation followed by complete paralysis, and complete paralysis alone (under deep anesthesia). The stage of paralysis of whatever kind was synonymous with concussion, and was reached at a threshold intensity which in the case of the movable head could be measured in terms of a threshold velocity at impact.

"Compression" concussion. Denny-Brown and Russell (15) drew a sharp distinction between the changes produced by a blow to the freely movable head and the effect of a sudden brief rise of intracranial pressure. They demon-

strated that passively applied pressures of 50 to 100 mm. Hg lasting 1 to 5 seconds had an effect which involved a prolonged fall of blood pressure, and initial acceleration of respiration followed by prolonged and often fatal apnea. This effect was uncertain, and similar or greater pressures often produced no effect. Herniation of the cerebellum and medulla at the foramen magnum was found in fatal experiments. On the other hand, an exceedingly brief impact of increased intracranial pressure induced by a blow on the plunger of a syringe evoked a concussion with loss of corneal reflex followed by gradual recovery, transitory immediate apnea, and small rise in blood pressure. As compared with "acceleration" concussion, the rise of blood pressure and the respiratory disturbance were slight in relation to the duration of loss of corneal reflex. The pinna reflex was found to be longer delayed in recovery than the corneal response. Reference to the records of Kocher (59), Cannon (9), Knauer and Enderlen (57), Scott (90) and others who, by striking the fixed skull, had used an injury which induced compression, revealed a similar absence or smallness of respiratory and cardiac response, as compared with those of Polis (81) and ourselves in accelerative injury. The condition resulting from very rapid brief compression was therefore distinguished as "compression concussion." It was pointed out that this also had a threshold value for elicitation which could be expressed in speed of application, though no attempt to measure it was made. By means of fitting to the pendulum a small piston which fitted a trephine opening in the fixed skull, and arranging the pendulum to bounce back from a metal stop after the piston had penetrated a regulated distance, we (15) showed that very severe compression concussion could cause intense apnea and prolonged rise of blood pressure, then indistinguishable from acceleration concussion, though small contusions of the brain stem were a common complication.

It is important to exclude the effect of herniation of the cerebellum at the foramen magnum and the production of lesions in the lower medulla and upper spinal cord in this way. Thus Githens and Meltzer (32) report as "concussion" with "unconsciousness," a condition of quadriplegia without loss of corneal reflex produced by means of a weight falling on the supported head, and associated with hemorrhage into the medulla and upper cervical cord.

Groat, Magoun, Dey and Windle (38) and Walker *et al.* (98) have since compared concussion produced by acceleration and by compression and found no essential difference in the sequence of physiological changes to result. Applied transient increase of intracranial pressure was found to have a threshold value expressed in total energy by the former authors, who also noted greater effectiveness if the cannula were connected to the posterior fossa. No studies of speed of application have been made. Gurdjian and Webster (40), (100) had difficulty in producing concussion uncomplicated by hemorrhagic lesions in dogs, but found no essential difference in the phenomena of response to a blow by pendulum to the movable head, the fixed head, or by penetration of bullet. In each case the degree of response could be graduated in terms of energy of striking force. Brief compression by transmission of fluid impact is therefore a convenient and reliable

method of inducing concussion. This does not however mean that the mechanism of "acceleration" concussion is necessarily identical with the effects of brief compression, or that concussion is the effect of a brief anemia of the brain. The hypothesis of Duret (23) which assumes that a wave of cerebrospinal fluid distends the ventricles, and related explanations, do not have further consideration since it has been demonstrated that concussion can occur after drainage of all fluid, and indeed in a decerebrate preparation when the cerebral ventricles have been removed.

The physiological mechanism underlying the generalized response. The experiments of Witkowski (107), Polis (81), Miller (68), White *et al.* (101) and ourselves have clearly shown that the mechanism of the physiological effects is independent of the occurrence of meningeal or petechial cerebral hemorrhage and of cerebral or spinal contusion. Examination of nerve cells of animals dying from such concussion or killed within two hours revealed no changes. The blood vessels of the brain are histologically unaltered. Denny-Brown and Russell (15) and White *et al.* (101) failed to find evidence of fat embolism which Gutiérriez-Mahoney (43) had claimed. Windle, Groat and Fox (106) have more recently also failed to find any histological change in such animals with the same methods by which they have demonstrated the remarkable delayed histological change which will be discussed below. Denny-Brown and Russell (15) were able to demonstrate that when petechial or meningeal hemorrhage or contusion did occur they developed in the course of one or more minutes following the injury when the chief effects of concussion had already subsided. The effects produced by such hemorrhages were traceable to any increase of intracranial pressure caused by them. Sensitive recording of intracranial pressure revealed no change in the first hours following concussion without lesions, except for a transitory rise of 2 to 3 cm. of water accompanying the immediate rise of blood pressure (15), or a slightly greater increase, attributed also to vascular congestion by Pilcher (80). Delayed effects, such as these described by White *et al.* (101), are not of account in the immediate phenomena.

If discrete lesions of the brain and cerebral edema are excluded as the basis for the immediate phenomenon of concussion there is next to be considered the possibility that the condition is determined by primary disorder in one part of the brain whence some secondary reflex or other effect affects the remainder. Concussion is mimicked to an extraordinary degree in the stilling of automatic behavior of the decerebrate "midbrain" animal. The disturbance of the medullary centers is then identical with those of the intact animal. Section of the VIIIth nerves excludes labyrinthine shock as a primary stimulus. The electroencephalogram has however given strong evidence that the cerebral cortex is primarily affected, independently of the bulbar centers. Williams and Denny-Brown (103), in cats lightly anesthetized with nembutal or ether, succeeded in showing a great loss of voltage in the electroencephalogram with progressive but slow recovery of wave forms. This change was independent of the apnea of concussion. Following abolition of the corneal reflex for 19 seconds in one ex-

periment the electroencephalogram showed only small voltage spikes for 7 seconds, very low voltage for 2 minutes, and fully recovered only after 20 minutes. Two and one-half minutes after the blow slow waves at one a second began to be prominent and remained so for some minutes. These very slow waves have not been commented upon by other investigators, though they are prominent in prolonged traumatic confusion (Williams, 102) and may represent this stage in more brief concussions. The effect was generalized, though the blow was given on one side of the head. If a heavy blow were given at one point in the skull, over one electrode, and the head prevented from moving, an identical but brief loss of voltage and progressive recovery occurred under that electrode and not elsewhere. These investigators therefore made a further point, that the electroencephalographic effect of physical impact can be localized, and the phenomenon seen in cerebral concussion is particular only in its generalization.

Dow and Raaf (22) had an exceptional opportunity for observing early human electroencephalographic changes in head injuries in shipyard accidents. Patients who had suffered an amnesia following the trauma but were clear mentally by the time the tracing was taken showed only a slight increase in the percentage of abnormal records as compared to a control series without loss of awareness. If there was impairment of consciousness of any degree at the time of the electroencephalogram the tracing was abnormal. The electrical changes are thus brief in most cases. In all cases the abnormality in electrical record was less reliable than clinical judgment in predicting time lost from work. Jasper *et al.* (56) and Williams (102) have studied the prolonged electroencephalographic changes that accompany prolonged traumatic confusion, and the latter author has pointed their resemblance to those of transient mild injury, and has followed their subsidence. They do not appear to be related to post-traumatic epilepsy, which is closely correlated with localized cerebral laceration (18).

Govons, Lewey and Grant (36) and Govons (35) recently reported a disorder produced by the explosion of a detonator attached to the parietal vertex in dogs, which differs considerably from the effects already mentioned. After an initial period of loss of reactivity lasting some minutes there was very slow recovery of reflex and general responses, after which period gross ataxia of gait and station become apparent and lasted 1 to 10 days. For days the animal was unable to land without falling, following a jump from one level down to another. The condition was reproduced in many animals, and in 4 of 12 surviving dogs no hemorrhages were demonstrable in the brain stem or cerebrum. A similar condition in a human patient was also described. Whether the ataxia was the result of parietal, cerebellar, or labyrinthine disorder is not clearly indicated by the experimental evidence, but the authors favor explanation by labyrinthine lesion. The labyrinths are to be reported upon later. It would appear necessary to postulate some localized intensity of the injury, so as to confine the most intense disturbance to one physiological mechanism. We have seen that the electroencephalographic change can be localized to one area of the cortex by a localized injury, and there would appear to be no *a priori* reason against localization of more severe or more persistent damage to one or other part of the brain by some

peculiarity of stimulus. It will be apparent however that in cerebral concussion, as generally understood, there is no evidence of localized effect.[3]

The possibility that generalized cortical changes in concussion result from some temporary ischemia of the cerebral cortex has been the basis of many hypotheses (59), (97), (90). The measurement of blood flow through the brain during and immediately following a blow to the mobile head offers a considerable technical difficulty. Methods involving drop recorders of jugular flow further involve the secondary effect of the loss of blood. With such recorders Polis (81) and Knauer and Enderlen (57) recorded a large increase in jugular flow accompanying the phase of rise of blood pressure, followed by diminished outflow. The former investigator provoked the injury with the head mobile, the latter with the head fixed. This was confirmed by Denny-Brown and Russell (15) by the same method with "acceleration concussion" and in addition these investigators found an increased outflow accompanying the small fall in blood pressure of "subconcussive blows." This increase in cerebral blood flow, associated with bradycardia and pause in respiration, appeared to derive explanation from stimulation of the depressor mechanism of the vagus by subconcussive blows, and has no essential part in concussion. With such a method a momentary pause commonly found before the increased rate of flow begins may have been a purely mechanical effect of the blow. The increased outflow was more prolonged in concussion when vagal effects are in any case also more prolonged. Polis found a momentary fall in blood pressure in the peripheral end of the cut internal carotid in some experiments, from which he postulated an initial spasm of the circle of Willis. This we were unable to confirm. The decreased cerebral blood flow one hour after trauma with decreased oxygen consumption, found by Lindquist and LeRoy (65), was in the presence of cerebral contusion which is itself a demonstrable alteration of cerebral circulation and should not be allowed to complicate discussions of cerebral concussion.

Reports of decreased oxygen saturation of arterial blood by Schnedorf *et al.* (89) following "brain concussion" take no account of the respiratory changes involved, and do not define criteria of concussion except to note that unconsciousness in one dog lasted two minutes, and that in all animals except one the spinal fluid was blood stained. Gurdjian and his colleagues (41) have recently made a careful chemical analysis of the brain, finding no change in cerebral a-v differences in oxygen, carbon dioxide or glucose content. They found an increase of oxygen saturation of arterial blood proportional to the disturbance of respiratory function. Their experiments were complicated by contusions but they note that some areas of the cortex were chemically normal even in profoundly injured animals and that the changes indicative of defective oxidation occurred in areas of marked contusion where they were proportional to the degree of local damage. The report of Krauer and Enderlen (51) of increased acidity of traumatized

[3] Labyrinthine concussion, as judged by the appearance of immediate and severe vertigo with progressive subsidence of that symptom is a very unusual accompaniment of head injury, and the post-traumatic dizziness commonly complained of is thought to have other origin (Friedman, Brenner and Denny-Brown, 30).

cortex to litmus is presumably related to the appearance of lactic acid, but its absence in uncontused cortex in the careful work of Gurdjian *et al.* indicates that it need no longer be taken into account in the mechanism of concussion.

It therefore does not appear possible that the cortical phenomena of concussion can be explained by the accompanying disorder of the bulbar mechanism. Indeed the immediate onset of unconsciousness and the peculiar phenomenon of retrograde amnesia completely differentiate concussion from syncopal attack or anoxia. The only comparable reaction in man is the confusion, often with retrograde amnesia, induced by cerebral electric shock, as now widely used in psychiatric therapy.

The electroencephalographic method as used by the author did not however allow any observation of the immediate electrical effect owing to blocking of the amplifiers for ten seconds by the impact contact. Walker, Kollros and Case (98) have endeavored to overcome this difficulty by the use of a voltage divider to stabilize the amplifiers. Concussion was induced by the fall of a weight onto water in contact with the dura mater in cats. They found a large spike potential (approximately 1 millivolt) to accompany the impact followed by a series of very fast spikes of the kind commonly seen in muscular activity for 10 to 20 seconds, and this in turn by a relative inactivity for a further period of seconds or minutes. Thereafter there was slow return to normal electroencephalographic activity. The animals were curarized in order to avoid the start reflex. An initial spike potential up to a hundred microvolts was found with the first repetition of the blow after death, thereafter no change. The effect of lesser blows than that estimated to cause concussion is not stated, nor is the early fast electroencephalographic discharge correlated with the other physiological changes. These investigators maintain that the primary event in concussion is an intense excitation of the nervous system, with activity which they term "after discharge" lasting up to 20 seconds, followed by a phase of decreased activity with a raised threshold to excitation which they categorically identify with "extinction." The disorder of consciousness is explained by analogy with the similar disturbance accompanying electrically induced convulsions in man. This view appears to be an oversimplification of the problem, for although a generalized excitation at the moment of impact occurs in lower level systems, and is likely in the cerebral cortex, the sequence of events from that point onward is more complex. A convulsive spasm is commonly seen in animals but after an initial flaccid delay of 5 to 10 seconds from the moment of impact. It is moreover usually a burst of running movement or intense general flexion, and tonic-clonic phases characteristic of cortical activity have not been seen by ourselves, though they are prominent in cerebral anoxia. In man there is no record of a convulsive fit with concussion, though the author has observed generalized extension of the limbs for a few seconds, with latency similar to animal experiment. An additional difficulty in the interpretation of the records shown by Walker *et al.* (98) is that the method of induction of "concussion" is such that it is necessary to eliminate residual compression. The magnitude and duration of pressure change from the

falling weight are not stated.[4] It would appear important to exclude prolonged ischemia of the cortex. The anesthesia, as Williams and Denny-Brown (103) remark, has to be extremely light. If the anesthesia were deep it was difficult to produce any prolonged effect on the electroencephalogram, although the paralytic phenomena of concussion on brain stem mechanisms were more severe and less complicated by excitation. There is no convincing evidence that "intense traumatic stimulation is followed by the same electroencephalographic, chemical, and clinical phenomena which characterize intense stimulation of the nervous system by electrical, chemical or other agents" (98), for in man convulsive effects are prominent in all these except trauma. Lastly, it may be likely that the neuronal disturbance of concussion is a universal phenomenon in nervous gray matter, and a phenomenon comparable to "extinction" arising from a single stimulus has yet to be demonstrated in medullary centers.

One of the most enigmatic features of concussion is the sensitivity of supranuclear mechanisms to the condition. Denny-Brown and Russell (15) in attempting to account for the complex sequence of events in the recovery stage postulated a paralysis of reflex transmission as explanation. Groat, Magoun, Day and Windle (38) by ingenious placement of electrodes by the Horsley Clark instrument succeeded in measuring steady thresholds of excitation for brain stem nuclei, supranuclear pathways and tracts. They found that either acceleration or compression concussion sufficient to abolish the corneal reflex raised the threshold momentarily in motor nuclei, and very considerably and for long periods in supranuclear pathways. Thus for basis pedunculi (white matter) there was no change in threshold during concussion but for motor cortex a rise in threshold from 3.5 volts to 7.5 volts. For facial nerve fibers near the genu there was no change but for the same movement (lid closure) from the neighborhood of the facial nucleus a sharp immediate rise from 0.8 volt to 2.5 volts was found. The effect was transitory, but could persist for as long as $1\frac{1}{2}$ hours before full recovery. It would thus appear that heightened reflex threshold is a general phenomenon, and the exact measurements of Groat *et al.* give sound basis for the hypothesis that each "center" is affected in the same way, but for a period which bears a fairly constant relationship to other centers. The transitory disappearance of the knee jerk and lower level reflexes no doubt reflects a similar change in the pontine reticulum. In very severe concussion the threshold of lower motor neurones was raised.

The physiological evidence appears now well enough established to conclude that the cerebral phenomena of cerebral concussion are the result of a cycle of events in each and every neurone mechanism within the cranium. The process is characterized by excitation followed by a refractory interval which varies from neurone to neurone, being most brief in the respiratory center and most prolonged in some portions of the cortical apparatus. Further, it appears to be

[4] Doctor Walker has informed the author that the significant pressure change lasted approximately 0.1 sec., and that a residual pressure of not greater than 2 to 3 cm. water remained.

certain that synaptic transmission exhibits the paralytic stage with a less intense injury than does the neurone as a whole. The relationship of the paralytic stage to "extinction" in the cerebral cortex is considered doubtful. Since the unique manifestations of cerebral concussion belong to the negative phase of neuronal activity, and it is the occurrence of this phase that determines human concussion, it appears preferable to maintain emphasis on the character of paralysis.

Persistent effects. The notion that traumatic edema may be responsible for some of the phenomena of concussion appears commonly in surgical literature. Cannon (9) showed in the cat brain that cerebral edema followed necrosis of cerebral tissue, but did not succeed in demonstrating any maintained or steady increase of cerebral bulk with a head injury in the absence of cerebral lesions. Pilcher (80) and ourselves (15) found no significant change in cerebrospinal fluid pressure in the first hour following concussion in the cat. The subject has been reopened recently by White *et al.* (101) who have shown by microvolumetric measurements of brain volume that in the cat an increase in bulk of the brain began 15 minutes after a concussion sufficient to abolish the corneal reflex for one minute and could reach as much as 5.5 per cent increase in 5 to 24 hours. The swelling was independent of any hemorrhage, and subsided within 3 days. Any rise of cerebrospinal fluid pressure was within 5 cm. water. The meaning of such a change is difficult to explain. The changes in blood pressure and respiration and other phenomena are past long before the swelling had commenced. Fat embolism was excluded. The condition does not appear to be responsible for any of the outward phenomena of concussion.

The weakness of what has been called the "molecular" hypothesis of concussion as opposed to those based on the supposition of vascular stasis, petechial hemorrhages, edema, anemia, etc., has been that there was no structural or comparable evidence that trauma could so disorder nervous function. Chromatolysis had been looked for by Jakob (56), by ourselves and by White *et al.* (101) without success. Jakob reported some small changes in myelin and occasional petechial hemorrhages at intervals of weeks after severe injuries, but none at intervals of one day after concussion.

This uncertainty has now been largely removed by the remarkable work of Windle, Groat and Fox (106) who, with a standard and controlled Nissl technique, examined the brains of guinea pigs at intervals of hours to days after carefully controlled concussion and recovery. They, like the preceding investigators, failed to find any immediate histological change, but describe a chromatolysis particularly evident in the large cells of the vestibular and red nuclei beginning 14 hours after injury, becoming clearly defined in 2 days, and progressing to complete dissolution of Nissl substance in some cells, between the 6th and 8th day. Disruption of the nucleolus was an early sign of the process. A maximum of 50 per cent of the cells in any nucleus was affected. Primary motor neurones of cranial nerve nuclei were seldom affected. They were unable to find similar change in the small cells of the guinea-pig cortex, but in later experiments in monkeys (39), where the larger cells permit better observation,

found the same changes in the brain stem nuclei, and in addition a different but definite chromatolytic change in scattered cells throughout the cerebral cortex. Here the change takes the form of a shrinkage of the entire cell with densification and fusion of Nissl substance. These changes did not occur after a subconcussive blow though two such blows in one animal produced a slight degree of change. The chromatolysis was slower in development than that of anoxia, more rapid than axonal reaction, and differed from these in the diffuse powdering of the granules in the early stages and in the lack of displacement of the nucleus. No changes were found in fiber tracts except some swelling of myelin beginning 2 days after concussion. They were of the opinion that these changes were reversible but a proportion of affected cells in all regions underwent complete lysis, though the evidence of this was only complete disappearance of Nissl substance without glial reaction. It is of great importance to determine whether the change is entirely reversible, or whether some cells can be destroyed by concussion, for the problem of traumatic dementia is a vexed one, and there is some evidence that concussion alone in a healthy person does not impair intellectual function even when periods of confusion lasting weeks have been survived (20). At present there is no clear evidence that a cell in which complete chromatolysis has occurred may not recover.

The chromatolysis pictured by Mott (71), (72) 12 to 24 hours after "shell-shock" and 8 hours after electrocution are not clearly enough portrayed to allow identification with the changes described above. The clinical states, as also in the case of Gutiérriez-Mahoney (43), are in addition insufficiently described and not sufficiently clear of the possibility of anoxia. The finding of Windle therefore stands as a unique contribution.

The question of the relationship of such changes to behavior remains. Both the edema described by White *et al.* and the histological change described by Windle *et al.* become clearly manifest only after some hours and thus may be interrelated. Such changes in human concussion have not been observed. Delayed rise in cerebrospinal fluid pressure is a rare event in the absence of contusion of the brain and not related to any particular circumstance. Prolonged coma and confusion are commonly but not necessarily associated with increased pressure (94), (16) and then usually in the presence of blood in the spinal fluid (70). It is considered that this association reflects only the necessarily severe nature of the injury, with greater likelihood of hemorrhagic cortical lesions. Whether the animals showing the changes described by Windle *et al.* were in a clinical state comparable to traumatic confusion remains to be determined. It may also be noted that in man the emotional and psychological effects commonly include fatigue and difficulty in concentration, though these frequently can be prolonged for many months in the presence of anxiety (20) and there is doubt as to the duration of histological effects for such periods. All the clinical evidence points to traumatic defect in intellectual function being maximal immediately after the injury and thereafter progressively declining, confusion being an intermediate stage. But we must not forget the peculiar behavior of the retrograde amnesia, and the elision of a memory of injury never to return. The

histological evidence of Windle and his associates indicates that the disturbance of concussion has delayed characters differing from any other known type of cerebral excitation.

Mechanism of injury. Most of the early work on the mechanism of cranial injuries was concerned with hypotheses of production of fractures. The physicist, learning that the brain is composed largely of water, will maintain that, at the speed of movement at which acceleration concussion occurs, the contents of the skull will behave as a solid. The physical factors then would become resolved into waves of compression traversing the cranium and cranial cavity. Ferrari (28) by demonstrating that glass threads placed in the brain were broken by a sudden impact sought to demonstrate that actual stresses occurred within the brain. Chipault and Braquehaye (11) with early Marey tambours showed that when the movable head was struck there was a momentary rise of pressure under the striking point and a momentary fall at an opposite point. We ourselves confirmed this. Kramer (60) demonstrated that the skull would bend under slowly applied force and showed an increase in internal pressure and Scott (90) found a considerable rise of pressure within the dog's skull when struck by a falling weight. Walker, Kollros and Case (98) have demonstrated the same phenomenon as did Chipault and Braquehaye with the advantage of the lesser inertia and fling of Hamilton manometers. They estimated the rise of proximal pressure and fall of distal pressure, each with subsequent fluctuations which by their different rhythms appear to indicate some periodicity in the contents of the manometer, rather than in the skull. Such manometric methods all have the disadvantage that the manometric inlets change the hydrostatic system. Even with the Hamilton manometer however it is inevitable that the manometer lead itself moves with the cranial wall into which it is screwed and thus imparts momentum to its contained fluid. It is also necessary to caution against the assumption that such pressures are widely distributed within the skull. Denny-Brown and Russell (15) found that when the manometer lead was placed to a point at right angles to the plane of impact the fluctuation was minimal or absent, and this is also discernible in the records of Walker *et al.* The brain must have momentum independent of that of the skull, allowing condensation on the side struck and a potential vacuum at the opposite pole (84).

Denny-Brown and Russell in drawing a distinction between acceleration concussion and compression concussion pointed to the difficulty in causing concussion of the fixed head without causing skull fracture, and the ability of a given blow, under certain circumstances, to cause cerebral injury in the mobile head while innocuous in the fixed head. The distinction has been misunderstood by subsequent writers (40, 98). The phenomenon, as was emphasized in the original paper, is strictly dependent on the mass of the striking object. With larger mass and slower velocity the reverse will obviously happen. The essential point at issue was that with small striking mass and high velocity the disorder was greater in the freely movable head when cranial volumetric distortion must theoretically be less. The situation with large mass and slower velocity

is beside the point, though of great interest when the extent of head injury as a whole is under discussion as in the work of Gurdjian and Webster (40).

We considered that acceleration and compression concussion, in spite of differences in causation, were representative of the same phenomenon in nervous structure, namely, the response to very rapid physical stress. Electroencephalographic studies have since then shown the same generalization of change throughout both hemispheres in both conditions. The Witkowski experiment with the heartless frog, the greatly increased cerebral blood flow with unchanged a-v O_2 and CO_2 differential, the lack of formation of lactic acid and the lack of histological evidence of anoxia are all strong evidence against any hypothesis of concussion based on the occurrence of transitory anoxia, ischemia, or primary metabolic change. The hypothesis of Kocher, using transitory compression as an explanation of cerebral anemia, has had to be discarded, not because compression of a special kind will not cause concussion, but because, as Denny-Brown and Russell maintained, the speed of impact has to be so great as to produce change of a different order from that of ischemia. Further, the phenomenon is generalized in acceleration concussion where one side of the brain is subjected to a positive and the other to a negative pressure.

The value of differentiating between the two types of concussion is first the rarity of injuries leading to compression concussion in civil practice, and the frequency of its occurrence in penetration of the skull by large fragments (15). Secondly, unless the differences are realized the physiological phenomena will not be fully understood. The statement of Walker *et al.* (98) that "it is possible by a blow to the head to cause concussion without producing any change in respiration or circulation" is comprehensible only if these authors had added that they refer to the fixed head, and thus to compression concussion.

Astonishing features of penetrating injuries of the skull are the infrequency of concussion, and the severity of medullary disorder in some of the few positive cases. Bomb and shell fragments traveling at high speed commonly traverse the brain without loss of consciousness. The only rational explanation would appear to be the lack of acceleration imparted to the head in such circumstances. With larger fragments, or with a 45 calibre bullet fired at close range, consciousness may be momentarily lost, or medullary contusion occur, or both (14). In this case the skull may have been thrown into motion as a whole, but the more probable explanation is the effect of such large fragments on intracranial pressure. In early writings this latter mechanism was thought to explain bursting fractures of the skull in these circumstances (11) and the effect was called the hydrostatic or explosive effect of a bullet. In connection with the causation of medullary contusion the same mechanism has been called a "plunger effect" by Denny-Brown (14), and also in relation to large depressed fractures in experimental penetrating injury (15). A recent paper by Webster and Gurdjian (100) demonstrates beautifully the effect of 22 calibre bullets in traversing the anterior fossa of the skull in dogs in causing the medullary symptoms of concussion, usually with additional hemorrhagic lesions. They showed the absence of symptoms if

the posterior fossa had been decompressed, and a correlation of degree of disorder with velocity of bullet. The 22 calibre bullet is relatively large compared to the size of the intracranial cavity of the dog, so that the size of projectile should be added to the factors they mention. The mechanism of action of such medullary disorder is that we have called compression concussion.

The question of movement of intracranial contents in head injury has been settled by non-instrumental means. In a series of experiments brilliantly conceived and as brilliantly executed a group of investigators—Sheldon, Pudenz, Restarski and Craig (91)—have devised a means of replacing the whole upper half of the skull of monkeys by a lucite calvarium. The dura is removed widely on either side of the sagittal sinus so that the brain can be observed in life. By means of high speed photography and a pneumatic plunger which produced graduated impact the behavior of the brain in concussion, and the effect of various directions and velocities of blow have been analyzed. In such a film shown by these investigators at the Annual Meeting of the American Neurological Association (1944) the effect of a lateral, temporal blow was seen to cause an indentation of the part struck followed at a perceptible but very small interval of time by sudden forward movement of the opposite side of the skull. The brain appeared to contract proximally with the impact and expand distally with the forward movement of the distal portion of the skull, following which a distinct but much slower swirling commotion of the brain within the cranial cavity occurred. These experiments also indicated that no remarkable anemia of the brain was part of the process and that considerable vasodilatation followed concussion. These investigators will report a detailed analysis of this phenomenon in due course, but for present purposes it may be taken that the rapid physical phase is a condensation of the whole brain, that is compression, then expansion, followed by movement about its attachments. Thus two possible physical mechanisms may occur.

It is remarkable that in acceleration a fall of pressure at one side of the brain and a rise of pressure at the other should produce a generalized phenomenon. Theoretical consideration of the hydrostatics such as those advanced by Goggio (33) do not offer assistance in this problem. Holbourn (50) has maintained on theoretical grounds that shear stress is more important than direct hydrostatic effect in the causation of concussion, and supports his contention by indicating the greater tearing of a gelatine model at points of partition when the model is rotated. Though this hypothesis has certain plausibility in relation to the common situation of pial contusion at the frontal and temporal poles in head injury, its author ignores certain important considerations. The most important of these (76) is that rotation of the head about an axis passing through the skull would be a peculiar origin of head injury, and that when the axis of rotation is outside the skull shear forces are minimal. Further, concussion in our experiments still occurred if the movement of the head was minimal (3 mm.) and purely postero-anterior. Finally, shear forces, by their tearing effect, must have predominant effect on the supporting vaso-astral framework of the brain, and it should now be clear that no case can be made for vascular lesions as a basis of concussion.

It is to be hoped that some means will be found to study the effect of distortions of structure (whether by rotational or linear shear stress) apart from circumstances of high speed compression-rarefaction, for there is now considerable evidence that all types of vascular lesions (including petechial hemorrhage) are independent of concussion both in causation and in effects. The effects of two types of movement seen through the lucite calvarium (91) could then be resolved with more certainty.

It is time that discussion of the mechanism of concussion be cleared of the confusing issues introduced by the focussing of attention on intracranial pressure by Kocher. We have maintained that compression concussion and acceleration concussion must have some common element of physical stress, although in the latter the pressure on one side of the brain falls at the moment of increase on the other. The factor common to both is condensation of the substance of the brain. All the evidence at present available supports a hypothesis by which concussion is a reaction to physical condensation of nervous tissue. Such condensation, like other kinds of nervous stimulus, requires a certain threshold rate of change to be effective.

The effects of the "blast" of explosives. The possibility that explosive force transmitted by air could induce some physical change in the central nervous system identical with or allied to cerebral concussion has been debated for a very long time. Accounts of military medicine in Napoleonic times discuss the possibility of some injury to the brain arising from the "wind" of a cannon ball close to the head (44), (21) and the discussions concerning "shell-shock" and "windage" in the World War 1914–18 are well known. Today the same subject is revived under the name "blast concussion" and Fulton (31) has given a general review of the problem. The term "shell shock" has been largely discontinued owing to the recommendations of a British War Office Committee (99) and a Ministry of Pensions Committee (69). These bodies recommended that clinical cases thought to be suffering from the transmitted effects of an explosion should be classified under recognized diagnoses such as psychoneurosis, cerebral contusion, concussion, etc., as became such states after more usual forms of head injury. There are many disadvantages that accrue from the loose application of a term with uncertain and even mysterious connotation in the eyes of the soldier (19).

At the outset of any discussion it should be clear that persons closely exposed to any explosion are liable to be injured by being thrown against some solid resistance, or by being struck by flying debris or portions of the explosive container. Thus, of a series of such cases, Eden and Turner (25) were able to account for all instances of unconsciousness in terms of such secondary injuries. The writer has related case histories from his own experience of exposure within 12 feet of bomb explosion with full memory of the injury although particles had lodged in the brain (17). In another case with a hysterical amnesia recoverable under amytal hypnosis the patient had been thrown into a heap of soft lime by the explosion. Both these varieties of injury are common within the experience of military surgeons. More difficult to assess are patients found wandering in a state of shock and confusion after exposure to an explosion without external sign

of injury and with resistant amnesia for the event. Unfortunately concussion is often sustained without sign of external injury in more usual circumstances such as a fall on a sandy surface. If such a condition as concussion from explosive force alone is to be established it is absolutely necessary to have full documentation of cases where all possibility of secondary injury is absent and amnesia both retrograde and immediate is verified. No such cases are on record.

The oft cited cases of Mott (71) do not withstand analysis, for the most convincing had been buried by an explosion, and the petechial hemorrhages found in the brain may have been due to CO poisoning from the fumes of explosion. In a further report Mott (72) cites two further cases. One of these had died suddenly following twenty-four hours of confused excited behavior, after exposure to many shell bursts. Multiple superficial petechial hemorrhages were found on the surface of the brain and there was a bruise of the scalp in the frontal region which suggests secondary injury. There was also a possibility of CO poisoning. The second patient had been sitting in a hut close to a large explosion and consciousness was not regained before death 12 hours later. Superficial hemorrhages were found over the cerebellum, temporal poles and one frontal lobe, with early and doubtful chromatolysis in the frontal cortex. The possibility of secondary injury was not excluded in this case also, and the changes found were those commonly seen in gross cerebral contusion. An interesting observation by Wilson and Tunbridge (105) on the occupants of a deep cave in Malta, at the entrance to which an aerial bomb exploded, describes the immobile apathy of the surviving occupants but gives no details of their disturbance of conciousness. The pathological observations are concerned with the pulmonary injuries alone.

The subject has been approached experimentally by a number of investigators. Carver and Dinsley (10) observed the effect of underwater explosions on fish, and describe a state of suspension of nervous activity which ended fatally in those in immediate proximity, and was followed by recovery after intervals up to 12 hours in those at a larger radius. At autopsy fish killed by the explosion showed petechial hemorrhages in the central nervous system, gills and viscera. Rats and mice were exposed to explosions in air, and animals showing no sign of direct trauma due to fragments of cage were rendered unconscious with bleeding from the orifices. "Varying amounts and degrees" of internal hemorrhage in the viscera and central nervous system were revealed. Some animals were found dead without visible macroscopic abnormality except general capillary engorgement especially noticeable in the nervous system. Animals exposed at greater distance were found in a state of "stupor," frequently with twitching of the limbs on recovery and exaggerated reflex activity. These animals also did not have lesions visible to the naked eye.

Marinesco (67) reported briefly on animals exposed to explosives, noting that there was no loss of consciousness, but that hemorrhage from the nose and mouth and ataxic gait was common. Petechial hemorrhages were found in the brain and spinal cord in many animals and the pulmonary lesion was well described.

Mairet and Durante (66) exposed rabbits to explosions in air. The animals

were either placed in trenches or suspended in air. Animals that succumbed were found to have pulmonary hemorrhages, small subpial hemorrhages on the spinal cord and nerve roots. The brain was without lesions in most instances but showed some petechial spots on the surface or in the pons. Those that survived showed acceleration of respiration and transitory myosis, and some were excited, some apathetic. The cells of the cerebral cortex were normal. After survival for long periods some unconvincing lesions were found, thought by the authors to indicate healed vascular lesions.

The very careful experiments of Hooker (52), (53) deserve particular mention. He exposed dogs at various distances to charges exploded on open ground, and to the muzzle blast of large rifles and howitzers. He describes the state of immobility and lack of reaction which these animals showed for periods of 4 to 6½ hours following the exposure. In no instance were the eyes closed, or reaction to stimulus abolished. The most striking effect was the sudden onset of "extreme fatigue." An animal previously alert, active and normal in every way was promptly changed into an exhausted lethargic animal which roused when spoken to or touched "and would move about heavily for a few moments at a time."

He likened the condition to surgical shock and measured the severe fall in blood pressure (to circa 50 mm.) found in every animal so examined. The abdominal veins were found to be distended at autopsy, but in no single instance was there evidence of macroscopic central nervous lesion. Fat embolism was sought for and not found. Pallor and other evidence of shock are commonly recorded immediately after exposure to explosion, and the experiments of Hooker indicate that this is not necessarily to be explained on an emotional basis. Hooker found pulmonary hemorrhage invariably associated with the condition of shock he described but under some circumstances when the pulmonary lesions were maximal the shock was least pronounced. He was therefore unable to derive an absolute correlation between the conditions. It is possible however that sudden traumatic compression of the thoracic cage is a strong vagal stimulus and no experiments to explore this possibility have to the writer's knowledge been made.

Zuckerman (108, 109, 110) and Krohn, Whitteridge and Zuckerman (62, 63) have repeated exposure of animals to air blast and have described the pulmonary lesions in detail. In goats and monkeys no cerebral lesions were found after exposure to blast pressures as high as 110 lb. per square inch (110). Though a ventricular or subpial cerebral hemorrhage was found in some rabbits, no condition of immediate traumatic unconsciousness was observed. Extradural spinal hemorrhage, and hemorrhages in spinal nerve roots were more common. In isolated instances of cerebral lesion the possibility of secondary cerebral injury from contact with the ground or cage, or of some embolic lesion from the pulmonary clots cannot be excluded. This difficulty also occurs in the report of a pheasant found in a dazed condition near a bomb crater by Stewart, Russell and Cone (92), where autopsy disclosed numerous petechial hemorrhages throughout the brain. Pathological reports on human cases dead of exposure to explosive

blast (26, 45, 75, 109, 63, 46, 47, 105) have all focussed attention on the pulmonary condition. Neither clinical nor autopsy evidence has indicated damage to the central nervous system. In only one case of blast injury, fatal in 4 hours, has autopsy revealed small submeningeal hemorrhages over the brain with a little blood in the ventricle (74). The immediate state of this patient was obscure for he appeared to have been asleep inside a hut which was demolished by the explosion.

The occurrence of visceral lesions resulting from exposure to underwater explosions has focussed attention on waterborne blast effects. A number of reports on clinical and pathological material are available (8, 34, 77, 48). Of these only Hamlin reports neuropsychiatric sequelae. In 35 cases examined by him one died of subdural hematoma which was however considered due to some local injury, probably from an earlier direct injury before immersion, one was disoriented with bloody spinal fluid, possibly from the same cause, and in the others there appears to have been no retrograde amnesia, though 4 of these suffered from some delayed loss of consciousness. None of the cases recorded by Goligher (34) lost consciousness or showed signs of damage to the nervous system.

The possibility of transmission of the impact of waterborne blast to the nervous system by the blood vessels (71, 92), or by the spinal fluid (48) has been suggested. Animal experiment (104, 37, 29) on goats and guinea pigs exposed to underwater explosions failed to demonstrate any evidence of central nervous lesions, and the animals were not rendered comatose immediately following injury. Observation of the reflex, cardiovascular and respiratory responses throughout such exposure was naturally difficult, and Clark and Ward (12) and Clark (13) overcame this difficulty by the use of a U-shaped tank, in the one limb of which the animal was immersed, in the other a float which could be struck by a falling weight. By this means these investigators demonstrated the mechanism of pulmonary and visceral lesions by the impact transmitted through water, even if a metal barrier prevented actual surge of fluid. Such an impact presumably travels with the speed of sound, and is of an estimated one five-thousandth of a second in duration. The mechanically induced wave appears to reproduce the effect of explosion in air or water, where transmission is of the same order. Hemorrhages visible to the naked eye did not occur in the nervous system. "Although occasional animals showed loss of corneal reflexes, ataxia and incoordination immediately after the blow, it was exceptional that any of these signs suggestive of direct and immediate involvement of the central nervous system occurred." No electroencephalographic evidence of concussion was found. Death could however be induced within 2 to 3 minutes. The impact was followed immediately by cessation of respiration and great slowing of the heart, effects which were abolished by vagal section. Re-establishment of labored respiratory movements within a minute was followed by gradual failure of respiration and heart beat if death occurred. Clark and Ward have thus established the chief characteristics of pulmonary and abdominal disorder by sudden impact. The close resemblance of the pulmonary and cardiac disturbance to that of cerebral concussion is to be noted. They investigated the effect of

immersing the head of the animal with and without exposing an area of dura, but were unable to induce cerebral concussion in rats or cats by this means. They remark that perhaps greater impacts might have induced cerebral concussion but since animals died within a few minutes of pulmonary hemorrhage with the blows used the problem of the influence of cerebral concussion was of minor importance.

The problem of cerebral concussion in blast injuries has therefore greatly diminished in importance following these clinical and experimental observations. Sudden death or coma following exposure to blast in air or water no longer necessitates explanation by a central nervous effect. Experience has revealed that loss of consciousness is delayed and not accompanied by retrograde amnesia. There remains however the occasional occurrence of petechial hemorrhages in both human and experimental observations (Mott, Marinesco, Carver and Dinsley, Stewart *et al.*, O'Reilly and Gloyne), or blood in the spinal fluid (Hamlin), and the occasional immediate loss of reflexes (Clark and Ward) or apathetic stuporous state (Hooker, Wilson and Tunbridge) or transient ataxia (Marinesco, Williams, Clark and Ward), to be explained.

Petechial hemorrhages in the brain can be caused by direct injuries but are then not usually generalized, as in Mott's cases, where the question of carbon monoxide fumes from the explosion was raised. Traumatic asphyxia, when widespread cutaneous, ocular and retro-orbital petechial hemorrhages are commonly found as a result of severe compression of the chest for 1 to 5 minutes (49, 5), and have occasionally been found in the brain in some cases where consciousness was not lost, appear to the writer to be a possible explanation in Mott's first case, where the patient was buried by the explosion, and perhaps in other cases of pulmonary contusion. The absence of cutaneous lesions would require some modification of the usual hemodynamic mechanism. Small terminal hemorrhagic spots within the nervous system are not uncommon in death from congestive heart failure and, as in the condition called traumatic asphyxia, the severe embarrassment of the venous circulation following pulmonary blast lesions may have a purely mechanical effect separable from the impact of the explosion. Robb-Smith (82) found fat embolism in some cases of severe multiple injuries from debris of bomb explosion, but these patients did not clinically resemble "concussion." An interesting case reported by Ascroft (2) presented two larger lesions which appear to the present writer to have been infarcts of the brain, as well as multiple petechial hemorrhages, following explosion of a "booby-trap" held against the chest wall. No trace of fat embolism was found. This case appears to raise a clear possibility of multiple emboli from the damaged lungs in these conditions and this should also be investigated. Hemorrhages in the spinal cord and roots have a more obvious explanation in distortion of the spine by the injury, and fracture of the spine (29) or paraplegia (64) have been observed in experimental animals. The negative pressure wave of blast may be supposed by some to have some particular effect, but this has been demonstrated by Latner (64) to induce the characteristic pulmonary lesion.

We have seen that the definition of cerebral concussion includes an immediate

loss of awareness. It is naturally difficult to exclude the absence of a momentary loss immediately following a devastating explosion. The state of apathy so clearly described in Hooker's animals however appears to allow no such possibility and was in them attributable to the related fall in blood pressure. Clark and Ward do not remark upon the blood pressure in their animals, but the other immediate effects referable to strong vagal stimulation, followed by severe obstruction of the circulation, presuppose a cerebral anoxemia of such rapidity and severity that loss of reactivity could well occur in some animals within three or four seconds of the impact. The adrenals were not histologically changed (Marinesco). The ataxia and inco-ordination described in animals were not such as to require necessary damage to a nervous pathway. The whole disturbance appears to fit a description of acute circulatory collapse or shock.

Concussion, or indeed any similar physical damage to nervous tissue resulting from the force of an explosion transmitted by air or by water, therefore remains an unproven condition. Though high acceleration of the head by explosion would appear to be possible, the fact that explosive blast is a movement of air and that such air has to accelerate the heavier, rounded head warns that the initial acceleration in the critical first half-millisecond is not comparable with that produced by a solid mass moving at a much slower speed. Explosive blast exerts its full effect when mass is small in proportion to surface area, otherwise it is deflected.

CONCLUSIONS

Cerebral concussion is a phenomenon which has not only important practical considerations, but also merits attention as a fundamental mode of reaction of nervous tissue. Though recent investigation has done much to clarify its essential nature there are many aspects of the subject which present unsolved problems. Some of the factors that determine the presence or absence of concussion in penetrating gunshot injuries to the brain are now explained. The separation of the effects of concussion and of multiple hemorrhages, and the greater understanding of the pulmonary lesions due to explosive violence have seemed to clear up many of the doubtful features of the effects of explosive blast. No convincing evidence is found to support the hypothesis that explosive blast can produce a cerebral lesion comparable to concussion.

The introduction of methods of reproducing standard degrees of the condition and of measuring both the quantity of injury inflicted and the amount of effect produced have opened the possibility of measuring its relation to nervous metabolism. Its effect on synaptic transmission and the delayed development of a cycle of histological changes are at present complete enigmas which invite investigation. Its relationship to shock, to fatigue and to the similar but different changes induced by anoxia are unexplored. The cumulative effect of repeated minor concussion would appear to offer a good starting point in such investigations.

It is maintained that in all such investigations the character of disturbance of nervous activity should be closely defined in order to avoid the confusion that

has arisen and will still occur if traumatic concussion is not clearly differentiated from other kinds of disturbance of nervous reactivity. In some respects it is necessary to keep the different kinds of mechanism of production clearly identified.

With the rapid increase in knowledge of the condition in recent years it is a question whether a more accurate definition of cerebral concussion can now be devised. As the condition is primarily of clinical interest it is unlikely that any definition not based on the disturbance of consciousness or awareness would be generally acceptable. Though the primary event may be an excitation of nervous tissue, it is necessary to emphasize the transitory loss of nervous function which is the essential characteristic. Not everyone who "sees stars" is concussed. A physiological definition should also take into account the occurrence of a similar and probably identical phenomenon in the spinal cord (51) and peripheral nerve (61). It now has to admit histological change but not vascular or hemorrhagic factors. There is as yet no certain indication that complete lysis of nerve cells can be a direct effect. With these considerations in mind we suggest that concussion can be defined as a transitory and reversible nervous reaction with *immediate* onset following physical stress of sufficient violence and brevity, and characterized by progressive recovery thereafter. In man, amnesia both retrograde and postgrade is its chief external sign, accompanied by a loss of reactivity which is its clearest feature in animals. The loss of activity affects all tissue subjected to such stress but the duration of paralysis varies with the complexity of the nervous mechanism as well as the intensity of injury.

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THE APPRAISAL OF NUTRITIONAL STATUS (NUTRITURE[1]) IN HUMANS

With Especial Reference to Vitamin Deficiency Diseases

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Forty years ago Chittenden began the preface of his book *Physiological Economy in Nutrition* with the following words: "There is no subject of greater physiological importance, or of greater moment for the welfare of the human race, than the subject of nutrition. How best to maintain the body in a condition of health and strength, how to establish the highest degree of efficiency, both physical and mental, with the least expenditure of energy, are questions in nutrition that every enlightened person should know something of, and yet even the expert physiologist today is in an uncertain frame of mind as to what constitutes a proper dietary for different conditions of life and different degrees of activity. We hear on all sides widely divergent views regarding the needs of the body, as to the extent and character of the food requirements, contradictory statements as to the relative merits of animal and vegetable foods; indeed, there is great lack of agreement regarding many of the fundamental questions that constantly arise in any consideration of the nutrition of the human body. Especially is this true regarding the so-called dietary standards, or the food requirements of the healthy adult. Certain general standards have been more or less widely adopted, but a careful scrutiny of the conditions under which the data were collected leads to the conclusion that the standards in question have a very uncertain value, especially as we see many instances of people living, apparently in good physical condition, under a regime not at all in harmony with the existing standards."

This statement was made four decades ago with special reference to the proteins. Today it applies very aptly to the vitamins. Much evidence has been collected in widespread efforts to determine the nutritional status of individuals and populations with respect to the vitamins. In the interpretation of such evidence much confusion has arisen, and from a given mass of observational data different workers frequently draw quite opposite conclusions. In this paper an attempt will be made to identify the causes of such disagreement.

One of the causes of confusion has been the use of ill-defined, and even paradoxical words (such as "sub-clinical" in describing disease). For the present discussion it is therefore desirable to define the chief terms to be employed.

Nutriture means "condition as to nourishment." It may be considered relative to one nutrient at a time, as is done below by the unqualified word; or relative to all nutrients, when it may be termed "general nutriture."

[1] Dr. H. M. Sinclair of the Oxford Nutrition Survey has revived the use of this obsolete word. The definition quoted above appears in the Oxford English Dictionary, and makes the word a useful one for present purposes.

Deficiency disease means any departure from health due to lack *in the body* of some essential nutrient present in foodstuffs. Deficiency disease can be due to one of two causes: (a) a *dietary deficiency*, i.e., use of a diet which contains an insufficient quantity of one or more essential nutrients; (b) an increased requirement above the usual level for one or more nutrients, due to what has been termed a "conditioning factor" (1, 2, 3).

Dietary standard means a statement of the amount of one or more nutrients which must be ingested in order to attain a given result (which always should be, but frequently is not, clearly specified).

Zones of nutriture. There are variations of nutriture from ideal to very bad; for this discussion we shall define five zones or levels of nutriture as follows:

1. *Saturation.* This is the state in which the body is incapable of increasing its content of a given nutrient even with prolonged ingestion of very large amounts. Presumably this should be nutriture at the highest possible level, except when the large amounts of the nutrient can work harm in the body. When this occurs one can speak of a zone of excess (e.g., hypervitaminosis A or D, obesity, or ketosis due to an excessive ingestion of fat).

2. *Unsaturated, but functionally unimpaired.* This level of nutriture signifies that the body contains less of the given nutrient than at saturation, but that no clinically manifest or potential deficiency disease exists, and no known biochemical or physiological test indicates any functional abnormality.

3. *Potential deficiency disease.* When the departure from saturation is sufficiently great and sufficiently prolonged, the next lower level of nutriture is reached, in which potential deficiency disease is present. Such potential disease is regarded as existing when in the absence of clinical evidence of a deficiency (a) a new stress on the organism will cause a rapid development of the clinically manifest disease or (b) a suitable physiological or biochemical test yields evidence of decreased reserve functional capacity. Reserve functional capacity is understood to be the ability of the organism to withstand a stress without deviation from its usual physiological course.

4. *Latent deficiency disease.* This is the mildest clinically detectable form of deficiency disease. It is characterized by vague, indefinite, non-specific symptoms. Hess' statement (4) regarding latent scurvy is applicable to all such states: "The diagnosis of latent scurvy is based mainly on the reaction to specific therapy and on marked improvement when orange juice, potato or other antiscorbutic food is taken. The symptoms themselves are suggestive but do not permit a definite diagnosis."

5. *Clinically manifest deficiency disease.* Such disease may be mild or severe. Mild manifest deficiency disease is that in which the classical syndrome has not appeared, but in which the symptoms and signs are highly suggestive, although laboratory tests and therapeutic trial may be necessary to establish the diagnosis. Severe deficiency disease gives rise to the classical syndrome and can usually be diagnosed without recourse to laboratory tests or therapeutic trial.

CONCEPTS OF THE DEVELOPMENT OF DEFICIENCY DISEASES. A widely held concept is that a deficiency disease develops in a series of stages, from a decreased

concentration of the vitamin in the blood, followed by diminished body stores and excretion, to a functional impairment, to microscopic and finally to gross anatomical changes. This is a rational hypothesis, used by a number of investigators as a starting point for further enquiry. The sequence of events is not necessarily the same for each vitamin, but some such sequence may be expected to occur, during which the nutriture changes from a condition of saturation, to unsaturation, to potential deficiency disease, to latent deficiency disease, and finally to manifest deficiency disease. This simple hypothesis has been extended by Kruse (5) to cover distinctions which he makes between acute and chronic deficiency diseases. The extended hypothesis is based upon data obtained by gross and biomicroscopic examination of certain surface tissues (conjunctiva, cornea, lingual papillae, and gingivae) which are assumed to be specific "indicators" of the nutriture with respect to four of the vitamins. This assumption is untenable in view of the weight of evidence against it (cited below, page 335); consequently the extended hypothesis based upon such observations is unacceptable until positive evidence supporting it is presented.

A deduction frequently made in the past is that for every manifest case of deficiency disease caused by severe dietary deficiency, many persons must consume diets deviating less widely from completeness and therefore be suffering either from mild, or latent, or potential deficiency disease. Many attempts have therefore been made (a) to refine the methods of clinical diagnosis of mild and latent deficiency disease; (b) to devise biochemical or physiological tests which will detect potential deficiency disease. Unfortunately these attempts have met with little success; at the present time there are few, if any, clinical criteria or functional tests which are widely agreed upon as reliable and specific for the detection of latent or potential deficiency diseases.

An interesting suggestion has been made by Carleen *et al.* (8) who postulated that for those water-soluble vitamins which enter into the molecular structure of coenzymes, the curve relating maximal rate of action of the enzyme to tissue concentration of the vitamin will be the well-known curve of dissociation of any enzyme. Assuming that failure of the enzyme to function rapidly enough is the underlying cause of a deficiency disease, one can then divide the curve arbitrarily into segments which correspond to the five zones of nutriture defined above. A slight dietary deficiency would cause the nutriture of an individual to sink perhaps to the third or fourth zone, and then remain unchanged. A more severe dietary deficiency might cause the development of manifest clinical deficiency in one of two sequences. First, the nutriture may deteriorate steadily, passing slowly down through the second, third and fourth zones, until the fifth is reached; second, the nutriture may change relatively slowly for a considerable time, and then plunge rapidly downward through the third and fourth zones into the fifth. There exists at present no evidence which enables us to choose decisively between these two views; in fact either sequence may occur, depending upon the conditions, or one may be characteristic of a deficiency of one vitamin, the other of deficiency of another vitamin. The first course would be compatible with the oft-quoted estimates (9, 10) of a high ratio of potential and latent deficiency diseases to mani-

fest deficiency diseases. But attempts in recent years to demonstrate objectively the existence of widespread potential and latent deficiency disease have met with great difficulty. This suggests that the ratio may not be high and so may support the second view against the first.

Mitchell (7) has recently reviewed the question of the ability of the organism to adapt itself to low intakes of essential nutrients and has suggested that dietary deficiency if not too severe may lead first to the less severe deficiency diseases, followed by an adaptation to the lowered intakes after which the signs of deficiency disease disappear. Definite adaptation to low intakes (intakes which by comparison with some recent dietary standards would be termed deficient) of calories (11) calcium (12) or thiamine (13) appears to occur. This view has also been discussed by van Veen (14).

MEANS OF ASSESSING NUTRITURE. Methods of assessing nutriture are (a) clinical examination, (b) demonstration of a biochemical or physiological lesion, and (c) estimation of the past dietary intake. When severe disease exists, the clinical examination is a sufficient tool. Attempts are constantly being made to refine this tool, so that it may become possible to detect mild and latent diseases with it, and with less reliance on other methods (15, 16, 17). For the detection of potential and latent disease reliance must be placed on therapeutic trial and on the detection of biochemical lesions.

Before such biochemical tests are considered established criteria of nutriture, their validity must be satisfactorily established by experiments on *human* subjects in whom unquestioned deficiency disease is produced, or in whom the spontaneously occurring disease is correlated with a low intake of the specific nutrient and with laboratory data indicating bodily lack of that nutrient. The disease must be curable by moderate amounts of the specific nutrient without other environmental changes. Such findings must be capable of reproduction by independent investigators.

The estimation of the dietary intake of the essential nutrients for the assessment of nutriture is of secondary value at present. For patients with manifest deficiency disease there is likely to be an obvious correlation with lack of the specific nutrient implicated. The method is too unreliable for the detection of potential or latent disease, due to lack of suitable dietary standards with which comparison can be made and to lack of knowledge of factors modifying the requirements for the nutrients. These difficulties vitiate the argument for "prevalence of inadequate diets as shown by surveys"[2] stated in Bulletin 109 of the National Research Council on "Inadequate Diets and Nutritional Deficiencies in the United States" (18). Other criticisms of that argument are: (a) The evidence obtained during depression years such as 1934 (19) yields no information about the dietary intakes of today. Most of the economically poorest classes of 1934 are relatively much better off now, and the consumption by them of better foods has risen sharply due to the improved economic condition and to educational campaigns. Ferguson and McHenry (22) have provided evidence of the influence of these factors over a two year period in their East York surveys. The

[2] i.e. *Dietary* surveys.

morbidity statistics for pellagra are of interest in this connection. In the eleven years 1933–1944 the number of reported cases of pellagra in North Carolina (20) was successively 833, 543, 732, 821, 576, 816, 214, 97, 60, 20, 15. Similar decreases have been observed in other Southern States (21). (b) Some of the data on which the argument is based are quite unreliable. For example the apparent very low intakes of vitamin C reported in Wayne County, North Carolina (18, tables 8–11), are much below the true intake, because when the estimates were made lack of knowledge of the vitamin C content of vegetables after the prolonged cooking generally used in this section led to the practice of counting zero ascorbic acid in all cooked vegetables. It has recently been shown (23) that this practice is quite incorrect, and that in fact much of the ascorbic acid ingested by the population studied does come from cooked vegetables. Therefore the earlier reported figures cannot be accepted by any critical reader as evidence of the true vitamin C intake of the population studied.

The results of determination of dietary intake may perhaps in the future be the indication for testing for potential deficiency disease just as today they are useful as supporting evidence for a diagnosis of clinically manifest disease, but not until the effect of given levels of intake of each nutrient has been observed in a suitable number of human subjects who are carefully watched for the development of clinical signs and symptoms. In view of the effect of various dietary patterns and of other environmental conditions on the requirement for each factor, it will be necessary to have a series of experiments to explore these influences, and to take account of them in diagnosis.

PREVALENCE OF SUB-NUTRITION. With the given definitions in mind, a discussion of some of the evidence which has been collected can now be profitably attempted. Evidence concerning each of the five zones of nutriture defined above will be given, in order.

1. *Saturation.* It appears to be widely recognized that few American subjects are saturated with any of the vitamins (18); nevertheless, some authors seek to define normality in terms of saturation alone, and imply that unsaturation unaccompanied by other signs represents a departure from normal (24). (This is discussed at greater length below.) From the incidence of obesity (25, 28) it appears that a fairly high percentage of the population is in the zone of excess nutriture as regards caloric intake.

2. *Unsaturated but functionally unimpaired.* It seems possible that the majority of the American population falls into this group, with regard to one or more of the vitamins. Certainly it has been demonstrated in many studies of apparently normal subjects showing no signs of disease (18), that many[3] are unsaturated with respect to vitamin C and complete absence of vitamin C from the plasma has been observed in a considerable number of subjects (26, 27, 28) in populations where scurvy among adults is unknown. It is to be recalled that in a case of experimentally produced avitaminosis C, the human subject showed departure

[3] Where particular cases are cited in support of an argument, it is important to remember that the condition described may or may not apply to vitamins other than that mentioned. Each will require separate investigation to establish the point.

from saturation (as measured by plasma levels of ascorbic acid) within a week of beginning to ingest a scorbutic diet, while the first evidence of derangement of function was not detected until after 3 months' consumption of this totally deficient diet (29). In spite of this, there has been a tendency to define scurvy in terms of lack of saturation with vitamin C; thus Abt and Farmer (30) speak of scurvy co-existing with plasma levels up to 0.5 mgm. per cent, and refer to a level between 0.5 and 0.6 mgm. per cent as indicating "beginning scurvy." Certainly these levels may represent unsaturation, but they are very frequently met with among quite healthy persons and there is reason to doubt whether scurvy or any sign of avitaminosis C appears at all while there is any vitamin C in the fasting plasma.

In this connection the findings of Levine, Gordon and Marples (31) are also instructive. In apparently healthy male premature infants they observed the excretion of *p*-hydroxyphenyl-lactic and *p*-hydroxyphenyl-pyruvic acids in the urine and the disappearance of these metabolites from the urine following the administration of ascorbic acid. The excretion of these compounds constitutes a biochemical lesion, and *this lesion could be eradicated by administration of amounts of ascorbic acid insufficient to raise the fasting plasma level of ascorbic acid above zero.* It is to be noted that these children did not exhibit clinical evidence of scurvy. In assessing observations reported in the literature it is essential to note what criteria were employed for the diagnosis of scurvy. (This caution should be generalized to cover all deficiency syndromes due to lack of other vitamins.)

3. *Potential deficiency disease.* The idea of potential deficiency disease is perhaps clarified by mention of examples. Among these we include the infants studied by Levine *et al.* who excreted abnormal end products of metabolism of tyrosine and phenylalanine; individuals who are free from clinical signs of thiamine deficiency, but who have a decreased ability to clear pyruvic and lactic acids from the blood following exercise or the administration of glucose; and such individuals as those in whom Follis *et al.* (32) found histological evidence of rickets at autopsy, although they may not have shown any detectable signs of rickets during life.

It is impossible at the moment to obtain any reliable direct estimate of the prevalence of potential deficiency states among the general population. The low incidence of clinically manifest deficiency diseases within the population leads us to infer that the reservoir of potential cases must be fairly small in number, or else the precipitating factors which determine the advent of the manifest diseases are almost totally absent.

4. *Latent deficiency disease.* The diagnosis of latent deficiency disease, as emphasized above, rests upon the presence of *non-specific symptoms*, upon knowledge of a probably inadequate intake, and upon a therapeutic trial. Laboratory data may be confirmatory. The symptoms are frequently those complained of by the maladjusted or neurasthenic patient. Ruffin (15) has pointed out that "vitamins . . . afford little relief in social, financial or domestic problems . . ." There exists a real dearth of reliable information about the prevalence of latent deficiencies,

because *all* the criteria mentioned above have not been applied to well-controlled groups.

5. *Manifest deficiency disease.* Mild deficiency disease has frequently been diagnosed from the presence of such signs as conjunctival changes, angular stomatitis, or changes in the appearance of the tongue. Such signs are not pathognomonic of specific deficiencies and may appear as a result of other causes (*vide infra*). At the present day there is very little severe deficiency disease encountered in this country, and the only one of the deficiency diseases to reach epidemic proportions in recent times is pellagra. Although morbidity and mortality statistics often are misleading, one can at least conclude from them that this disease is now quite uncommon. Experience of trained observers at such centers as Duke Hospital and Vanderbilt University Hospital show that it is now difficult to obtain even a few cases for study. The prevalence of mild manifest deficiency diseases is a matter of great doubt, since the diagnostic criteria are not generally agreed upon. From this it might be inferred that such diseases are by no means common, or they would have been recognized.

Before leaving the question of the prevalence of sub-nutrition, one reason for widely varying estimates of the incidence of deficiency diseases must be mentioned; this is the difference between the samples of the population encountered by different observers. In conducting a nutrition survey the investigator should either see the whole population of the chosen region, or a statistically representative sample of it. The investigator who follows either of these courses is likely to record a relatively small incidence of deficiency disease. On the other hand, the physician in a hospital out-patient clinic within the same region sees a highly selected sample which is not at all representative of the whole population. Such a sample is biased for its economic class and also for its consciousness of illness, and will present a relatively high incidence of deficiency diseases. This may well be the true incidence in the sample observed, but not of course *in the population at large.* The same criticism applies to those surveys which are carried out on selected economic groups; one cannot generalize from the results of surveys of such groups to the population at large.

DIETARY STANDARDS. A dietary standard is a statement of the amounts of one or more nutrients which should be ingested in order to attain a specified result. As Leitch (33) has pointed out, dietary standards have undergone evolution. They have indeed evolved from early ones designed only to supply sufficient aliment to keep body and soul together, to some present-day standards designed to realize to the limit man's material capacity for life, and perhaps even to improve his moral condition as well (34). Those most in use at the present day are generally designed either as minimal standards to supply sufficient of the vitamins to ward off all signs of deficiency disease (35), or as so-called optimal standards, intended to maintain health at a higher level than a minimum standard can do (36).

It is not yet certain whether this distinction between good nutrition and optimum is valid. Many workers in the field accept the distinction, but others consider that there is not yet good evidence to establish its existence (37, 38). It is instructive to consider it in terms of nutriture. The minimum standard must be

reached in order to attain the second zone of nutriture defined above, and the "optimum" standard would then raise the nutriture to the first zone. The first question to be decided is, does saturation produce a degree of health and resistance to disease greater than unsaturation? There exist no satisfactory data to answer this question, and there is wide divergence of opinion about the answer which will ultimately be obtained. We incline to the opinion that beyond an intake which will prevent potential deficiency disease, further increases in quantity of water-soluble vitamins ingested will confer no additional benefits upon the organism, except perhaps the ability to survive longer periods of deprivation. Even this benefit is not always realized, as was clearly demonstrated for vitamin C in the guinea pig by Zilva (117). For the fat-soluble vitamins this is not true, since such massive storage of these factors can occur. When in the course of experimental work an improvement in the condition of man or animal is seen as a result of additions to the diet, it is to be concluded that before the addition was made, the diet did not reach a minimum standard for the prevention of potential deficiency disease.

A most important point which is not always kept in mind regarding dietary standards is that they give a single figure (comparable to a mean value) as a measure of a quantity varying over a range even for individuals of the same sex, height, weight, age and activity. *Since the dietary standard possesses the properties of a mean, its usefulness as a yardstick for assessment of the mean intake of a nutrient by a population is considerably greater than its usefulness when applied to evaluation of the intake of an individual.* Ideally the standard should be based on experimental determination of the requirement of the nutrient for maintaining the individual in that zone of nutriture which the standard is designed to attain. In that case, it will be calculated from the *mean* of the observed values and will be accompanied by an appropriate standard deviation for the group, which can be used to evaluate the variations from the mean in any population whose intake is compared with the standard.

It must also be remembered that dietary standards for the various essential nutrients are to some extent interdependent, and therefore hold for only a single dietary pattern. Thus the standards for the vitamins B are now generally set relative to the caloric intake. There is evidence (25) that the Recommended Dietary Allowance (36) of calories is too high, and it is therefore probable that the figures for the vitamins B should be scaled down accordingly. The requirements for thiamine also depend upon the fat content of the diet (39); these have been discussed recently by McHenry (40) who has concluded that "the allowance for thiamine now commonly recommended is much too large and that, because of the use of this excessive allowance as a dietary standard, a great deal of the alleged deficiency is non-existent." Holt (41) has also recently concluded that the thiamine requirement on a diet chosen naturally lies between 0.17 and 0.23 mgm. per 1000 calories. (This would mean about 0.5 mgm. thiamine daily for a sedentary man.)

There is unfortunately a tendency to estimate the incidence of *deficiency diseases* by applying a dietary standard to the evaluation of measures of food intake

by the population at large. These estimates are likely to be very misleading, even when the intakes are accurately known, if they are interpreted with reference to a standard such as that of the National Research Council which is aimed at maintaining an "optimal" state of nutrition. Although such an interpretation is unwarranted and is not in keeping with the original purpose of the Recommended Dietary Allowances, much of the evidence for the existence of widespread potential and latent deficiency disease in the United States has been obtained in this way (18).

Another unfortunate circumstance is the very small amount of evidence upon which most of the dietary standards are based. Few human beings have ever been maintained on a deficient diet with precisely controlled quantities of one or more nutrients for a sufficient length of time to establish the intake levels at which deficiency diseases develop. Recent investigations suggest the possibility that the biosynthesis of thiamine (42), riboflavin (43) and nicotinic acid (44) occurs in some humans and therefore the minimal dietary requirement must vary greatly from one individual to another depending upon whether such "refection" is occurring and to what extent. With some of the vitamins, standards have been estimated in the complete absence of measurements on human beings, using as a basis only fragmentary data on other species (45). As a result, the tendency has been to make a high estimate of the actual mean requirement to prevent deficiency disease, then to make an extra allowance as a safety factor, and a further allowance to convert the "safe" minimal requirement into an "optimal" allowance. In such ways standards are devised which are very likely a good deal too high; the National Research Council standards can be criticised on these grounds.

Even for calories, about which we know far more than for any of the vitamins, and of which either an excess or a deficiency is harmful, there are suggestions that the figures may be too high. For instance, Milam and Anderson (77) consider 2000 calories the "general level of adult caloric intake" in a North Carolina population, and Youmans *et al.* (25) have reported similar findings. Winters and Leslie (46), by actual analysis of duplicate meals found that among 24 women of a low-income group in Austin, Texas, the average intake was only 1145 calories per day. It might seem desirable to judge the adequacy of caloric intake of an individual by his weight and its variations, and to restrict the use of *dietary standards for calories* to evaluation of mean intakes of populations.

THE CONCEPT OF NORMALITY. Ivy (47) has pointed out four different ways in which the word normal is applied to the structure, function and physico-chemical characteristics of the human body. One meaning, the "normative," requires that a normal person be one who is perfect according to some fixed set of standards. All others are regarded as abnormal (in nutritional condition they are the deficient). Such a meaning is used by Kruse in his discussions of nutritional problems, but as stated by Ivy "This view is obviously arbitrary and the authoritativeness of its standards may in many instances be questioned." This appears to be particularly true in nutrition work.

The second ("clinical") meaning defined by Ivy "maintains that anyone is normal who is well and not handicapped by some disturbance manifested by

symptoms. It views the mild imperfections of the body as normal." The remaining meanings are statistical: "arbitrary statistical," which involves observation of a homogeneous population and definition of that portion of the population as abnormal which deviates from the mean by more than a specified amount; and "non-arbitrary statistical," which "acknowledges no predetermined judgment and no arbitrariness." This last view maintains that "a sharp distinction between normal and abnormal does not exist for a group or even an individual. . . . It recognizes that degrees of normality and abnormality exist." It is likely that the clinical or the non-arbitrary statistical meaning of the word normal will be the most fruitful to choose for application to nutrition work, both in order to advance our knowledge, and as a basis for the solution of practical nutrition problems.

A slight departure of bodily structure or function from ideal, not causing illness or curtailment of the activities, work or happiness of an individual, and only detectable by histological examination either during life (e.g., biomicroscopic examination of surface organs) or post mortem, should not be regarded as a *significant* abnormality. Indeed it might be argued that some such departures indicate the adaptation of the individual. It follows that the claims for startlingly high incidence of deficiency diseases diagnosed on the basis of such departures from the ideal are to be heavily discounted. For instance, histological studies of bone at autopsy have been claimed to show that 46 per cent of a group of 450 children dying in hospitals had suffered from rickets (32); biomicroscopic examination of a group of 49 people was said to reveal that all showed lesions characteristic of nicotinic acid deficiency and also lesions characteristic of ascorbic acid deficiency (48, 49). Comparison may be made with the presence of simple lentigo. If one chooses the non-freckled individual as normal, all persons with lentigo would be termed abnormal. No one however would claim that such a condition would significantly affect the health of the individual. One cannot merely say "normal" or "abnormal," but must define the abnormality in terms of some measurable sign and demonstrate the significance of such abnormality.

The hemoglobin level affords a simple example from laboratory measurements pertinent to this discussion. It is frequently stated that the normal value for an adult female is 14 grams per 100 cc. of blood. This is a normative view. It implies that a subject with any lower value is abnormal and classes her as anemic. It classes as anemic the woman with a hemoglobin of 12 grams per cent who judges herself, and is judged by her physician, to be in excellent health; and yet there are such women, whose hemoglobin cannot be raised permanently, or sometimes even temporarily, to the level of 14 grams per cent by therapeutic measures (50). Such women should not be labelled anemic or abnormal, and their existence brands the normative concept as ill-considered in its generalized application to health problems.

THE QUESTION OF SPECIFICITY IN DEPARTURES FROM NORMAL MORPHOLOGY SEEN IN SOME DEFICIENCY DISEASES. The question of specificity is acutely raised by the use of such single slight deviations from ideal, unaccompanied by any other signs, as diagnostic of deficiency diseases when such deviations are found in large

proportions of the population. That is, are such deviations caused by the nutritional lack which is diagnosed, *and by that alone?* (In Ivy's terms, is the normative standard which has been employed authoritative?) Kruse has maintained that the changes resulting from a deficiency are tissue-specific. Thus, he characterizes aniacinosis by tongue changes (48), avitaminosis A by conjunctival changes (51), avitaminosis C by gingivitis (49), and riboflavin deficiency by corneal invasion (52). A review of the published reports of other investigators fails to substantiate such a claim. Thus, experimental gingivitis has resulted from a deficiency of vitamin A (53, 54), of vitamin D (55, 56), of vitamin M (57, 58, 59), of nicotinic acid (54), as well as ascorbic acid. Furthermore, one may find scurvy without gingivitis (29; 102, p. 89), gingivitis may develop after other signs of the disease (102, p. 177), and, finally, gingivitis as it commonly occurs in an otherwise healthy group of individuals may not respond to vitamin C therapy (60). Jeghers (61) has reviewed the multitude of deficiencies and other diseases which may produce abnormalities of the tongue and has wisely cautioned that "Pellagra glossitis or ariboflavinotic tongue should not be diagnosed unless there is other supporting evidence for these specific deficiencies, or unless the tongue is unusually characteristic."

While it is well established that some cases displaying Bitot's spots yield rapidly to therapy with vitamin A (62), other cases are resistant to treatment with this vitamin (63), and may exhibit plasma vitamin A levels within the range which is usually encountered in healthy individuals (79). Berliner (64) has questioned the diagnosis of xerosis conjunctivae as depicted by Kruse (51) as evidence of vitamin A deficiency. Darby and Milam (28) have found that the incidence of conjunctival changes described by Kruse was equally great in a population with a calculated intake of vitamin A of more than 2500 I.U. daily as it was in a population with a daily recorded intake of less than 2500 I.U. Finally, Kirkpatrick (65) noted in 1922 that night blindness was the first premonitory sign in what has now been established as clinical vitamin A deficiency. Keratitis and corneal invasion may unquestionably be produced in rats by riboflavin deficiency (66, 67, 68), and some of the spontaneously occurring vascularity of the cornea in humans yields to riboflavin therapy (69). Similar changes, however, have been observed in the following deficiencies: vitamin A (70), tryptophane or lysine (71), zinc (72), sodium (73), and some unknown factors (74, 75). Likewise, corneal invasion occurs in various diseases of the eye such as trachoma. It has not been possible to correlate the dietary intake of riboflavin with the occurrence of corneal vascularity (28, 76, 77), and therapeutic response to riboflavin in many instances has not been successful (78, 79). It must be concluded, therefore, that there exists no specific tissue alteration which alone may be considered pathognomonic of a single deficiency state. Furthermore, the basic question of what constitutes a departure from normality in the tissues examined by Kruse in his attempts to diagnose deficiency diseases is by no means a closed one.

THE POSSIBILITY OF OPTIMUM NUTRITURE. The use of the term optimum requires careful consideration, as it has generally been used without any clearly de-

fined meaning. The definition of optimum health is difficult; and the definition of optimum nutriture is perhaps still more difficult. How is optimum nutriture to be recognized? By size of the adult? By rate of growth? Not alone, for "a child may be destined to be tall or short; to progress rapidly or slowly through physiological time" (80). Furthermore, quantity does not insure quality, and there are disadvantages to the race in increasing the size of our bodies very far (81). By length of life? Only if we can measure its quality. Increased length of life, seemingly, may be promoted by more abundant intake of some factors, such as calcium (82, 83, 84, 85) or by a limited caloric intake (86). By resistance to infection? Here one must specify the type of infection, for it appears that while good nutriture may aid in warding off the effects of some bacterial agents (58, 59, 87) poor nutriture is a positive help in repulsing certain virus infections (88, 89, 90, 91). Aycock and Lutman (37) have recently reviewed the rôle which nutrition plays in warding off infection and have concluded "that vitamin deficiency as a factor in susceptibility to infection is not a general epidemiologic principle. The indications are that only deficiencies of certain vitamins affect susceptibility to certain types of infections and that these occur only in limited areas where these vitamin deficiencies reach a sufficiently severe degree to produce tissue changes which are favorable sites for secondary infection."

The term *optimum* has frequently been employed as synonymous with *maximum* when applied to nutriture. It is evident to all that such a concept is totally unacceptable as regards caloric intake. Thus obesity, which in all cases must result from a caloric intake in excess of the requirements of the individual (92), is associated with increased morbidity (93, 94) and increased death rate (95). Chittenden (96) expressed his concept of optimum nutrition as follows: "It is self-evident that the smallest amount of food that will serve to keep the body in a state of high efficiency is physiologically the most economical, and hence, the best adapted for the needs of the organism. Any excess over and above what is really needed is not only uneconomical, but may be directly injurious." Nicholls and Nimalasuriya (12) expressed a similar view: "A sufficiency of any constituent in the diet of healthy individuals is the optimum, nothing is gained by an excess, and as a general principle, dietary excesses should be avoided."

It appears that the term optimum has been used with a multitude of meanings, and that the meaning may vary for almost every user. It may well be argued that such a term should be expunged from the literature; and it does not appear to the writers to be one which can at present be of service in the advancement of nutritional science. A more valuable concept seems to be the definition of the two highest zones of nutriture which we have defined above as adequate or good, and the abandonment for the present of any distinction between optimal and adequate nutrition until a satisfactory meaning can be given to optimal.

The whole question of optimum as distinct from normal or adequate nutriture was most clearly discussed by Mitchell and Hamilton 15 years ago with respect to protein intake, and their conclusions may apply equally well to the intake of the various vitamins. They wrote (38, pp. 569–571):

"Evidently the postulate of an optimum nutrition superior to merely adequate

nutrition cannot be substantiated until a most searching and successful study of nutritive requirements has been completed, in order to prove that the condition of adequate nutrition with which the optimum is to be compared is in truth adequate. . . . It may seem that the whole question at issue here is merely one of the definition of terms, in particular the substitution of the word "adequate" to cover what is frequently referred to as "optimal." This substitution, however, replaces an indefinite term, appearing to involve implications difficult of acceptance, by a term perfectly definite in its significance. . . . The prevailing dietary habits in this country, for no obvious reason at all, are considered merely adequate; hence the possibility of increased human efficiency and happiness following super-adequate nutrition. . . .

"The great significance attached by Sherman to the marked improvement in the nutrition of rats induced by increasing the proportion of milk in the diet, is greatly diminished when it is realized that the inferior ration, containing the smaller proportion of milk solids, cannot fairly be called nutritively adequate, except insofar as it is capable of promoting *continuously* the different functions of animal life. However, the extent to which these functions operate should also be considered in assessing the adequacy of any dietary regime, i.e., adequate nutrition implies something more than continued health and existence. The experiments of Sherman simply afford an illustration of the different degrees of adequacy of different rations, and not of a super-adequate nutrition following upon increases in the consumption of milk."

If this point of view expressed by Mitchell and Hamilton had been carefully studied, much of the present day confusion and misconception about optimum nutrition need never have arisen.

THE QUESTION OF SLIGHT CHRONIC DEFICIENCY DISEASES. A point requiring further investigation is the rate of disappearance of minor changes in surface tissues resulting from chronic slight deficiency in the diet. According to Kruse (5), such lesions require months or years to cure. As mentioned above, there are good reasons to doubt the specificity of the lesions studied by him. Furthermore, one of the most striking things ever noticed about severe deficiency diseases involving just those tissues which are the site of such changes is the rapidity with which cure and restoration of the tissues follows specific treatment with small amounts of the missing factor (15, 17, 62, 97, 98, 99, 100, 101, 102, p. 236). Yet it is claimed that large intakes of the appropriate factors will cure only a proportion of subjects of their slight chronic deficiency states in a year (48, 49, 51). The simplest conclusion from this is that the refractory conditions being treated are not specifically due to deficient intake, and that the tissue changes seen over such long periods (necessarily involving change in environmental conditions) are due to other causes than the addition of the one factor to the diet. Moreover Ruffin has observed (103) that when a chronic mild pellagrin has had a denuded red tongue for a long period, nicotinic acid therapy induces just as rapid a cure and regeneration of the papillae as occurs in the recently affected tongue of an acutely ill pellagrin when he receives similar treatment. Satisfactory evidence of widespread mild chronic deficiency diseases which can only be very slowly

cured has not yet been obtained, and it appears to be reasonable to doubt that it will be obtained in this country under existing economic circumstances.

The claim has been made that a great deal of ill-health and incapacitation due to chronic latent or mild deficiency diseases occurs in this country (104, 105), but again the evidence to establish the claim soundly is wanting. To cite once more the pellagrin, as seen in North Carolina and Tennessee, there is little doubt that the acutely sick pellagrin has often suffered from a chronic latent deficiency of nicotinic acid for a considerable time, and that he is only precipitated into the acutely sick stage by an additional trauma. Frequently the farmer becomes acutely ill after a long day's exposure to the intense sunlight of early summer while working in his fields (106). In other words, he carries on his usual activities through the chronic latent or mild stage, and is only incapacitated after the onset of the acute severe disease.

Exactly the same sort of thing has been reported by Platt and Lu (100) for one type of beri-beri seen in the Orient. The patients continued their ordinary activities without difficulty and then suddenly collapsed and without specific therapy died in a few hours. Until much more objective and precise evidence appears, there is no reason to doubt that the same holds true of deficiencies of the other vitamins.

Experimental evidence bearing on this point must be most carefully controlled because it is likely to be based on subjective symptoms and reactions of the subjects, who may be abnormally confined and restricted or overtaxed by the burden imposed during periods on test diets. The use of large numbers of subjects and really adequate controls will help to prevent erroneous conclusions from being drawn. Muench (107) has emphasized the need for particular care in design of experiments in this field, and has mentioned some criteria which are often neglected.

Marrack (108, pp. 52–3) has epitomized the difficulty of interpretation of the evidence of slight deficiencies among the population at large—"With human beings it is not so easy to be sure about these minor deficiencies as they occur in those unplanned experiments on human beings in which the level of the diet is lowered by force of circumstances, economic or otherwise, or in response to taste or custom. Not only may the food be inadequate in several respects, but other unfavorable conditions, such as bad housing, may operate at the same time. The symptoms may be ill-defined; usually they may be attributed to several possible causes. Specialists in the appropriate branches of medicine would probably be able to incriminate a complex, a hormone defect or a surgical condition. People may get better without treatment or with any form of treatment. Before concluding that any *symptom* is due to deficiency of a particular vitamin, we should insist that the incidence of this symptom should be related to the amount of this vitamin in the patient's diet and that there should be a higher rate of recovery among patients treated with this vitamin than among control cases. The most satisfactory information is that obtained from the few deliberate experiments in which the vitamin supply of human beings has deliberately been limited."

THE DIRECTION OF FUTURE ADVANCE. One of the advances which will greatly facilitate the detection of the potential and latent deficiency diseases is the refinement of existing biochemical and physiological tests, and the invention of new and more delicate ones. At the present time very few of these tests are really reliable for diagnosing a latent deficiency in an individual. The visual threshold measurement at complete dark adaptation is an example of a useful procedure of this sort. This gives the earliest hint of vitamin A deficiency, *but only if* a raised threshold is quickly lowered following specific therapy (109).

Some progress is being made with other tests. It may be claimed that several are already of real value in the detection of potential and latent deficiency diseases, although in general they are still too cumbersome for widespread application. Attempts to simplify such tests sufficiently to make them applicable to nutritional surveys of populations are already meeting with some success. Examples of such tests are the measurement of ascorbic acid in the white cell-platelet layer (110); the rate of disappearance of blood pyruvate following exercise, as a measure of thiamine deficiency (111); fasting urinary excretion of thiamine, riboflavin and nicotinic acid (112); and simplified tests for the excretion of thiamine, riboflavin, and nicotinic acid metabolites following the administration of a fixed dose (113). These can be used to demonstrate differences between the average responses of different groups, but are of much more equivocal value in estimating the nutriture of the individual. This arises from two causes, the variation between different individuals which occurs for all attributes of living organisms, and the difficulty in fixing a level of response below which with a stated probability a deficiency disease will co-exist. This latter difficulty will persist until such tests can be carried out at intervals on subjects maintained on deficient diets long enough for the severe deficiency disease to appear; or alternately in the "experiments of nature" in which the severe disease develops on the usual diet of the individual. (Such subjects are unavailable in sufficient numbers in this country today). Only then will it be possible to evaluate the results of the tests in terms of clinical condition. An interesting example of this is provided by attempts of investigators in the U.S.A. to fix a "lower limit of normal" for the plasma vitamin A. Estimates have varied from 75 I.U. to 130 I.U. per 100 cc. (114). Recent work in China by Hsu (115) has demonstrated that patients with clinically manifest avitaminosis A had a mean level of 6 I.U., while his normal controls had 54 I.U., and hospital patients without clinical avitaminosis A had 36 I.U. Thus it appears that in this country there had been a failure to set a level below which deficiency disease occurs, simply because of the rarity with which such disease had been encountered. This conclusion is strengthened by failure to find evidence of impaired dark adaptation in various districts (116). Almost everyone here appears to have a sufficient supply of vitamin A, so that no evidence could be obtained about plasma levels of deficient people.

It is well to bear in mind the distinction between three types of biochemical test: (a) measurement of vitamin levels in the blood or urine of the untreated subject; (b) similar measurements for the subject after oral or parenteral administration of the vitamin ("load" or "saturation" tests); (c) detection of metabolic

products in the blood or urine which do not usually appear except as a result of a deficiency (functional test). It is possible to have a low level of a vitamin in the blood, or a low excretion in the urine, and yet have no functional impairment (31). This is in accord with the findings of Zilva (117) in work with the guinea pig. He demonstrated that urinary excretion of vitamin C occurs only after the tissues have been saturated with the vitamin. It seems possible then that the measurement of urinary excretion of vitamin C by the untreated subject merely determines his intake in excess of that which can be utilized or destroyed by the tissues. It is also possible to have a low level of vitamin C in the blood and yet to respond to a load test in the way a normal subject responds. Kajdi *et al.* (118) have reported one of the very few studies in which a load test has been correlated with presence or absence of manifest deficiency disease. The essential point of their test was the measurement of plasma ascorbic acid 4 hours after intramuscular injection of 200 mgm. of ascorbic acid, and the result was reported together with the observed signs for presence or absence of scurvy. The conclusion was that 4 hours after the injection values below 0.2 mgm. per cent occurred with clinical scurvy, and values above this level indicated absence of scurvy. Unfortunately this test does not appear to have been followed up by any other workers in the field.

For several years the sum of the evidence has suggested that fasting blood plasma levels and excretions of ascorbic acid following test doses have little relation to bodily requirement of the vitamin. Such measurements locate the nearness of the body to saturation with the vitamin, which is probably irrelevant. In a recent review Pijoan and Lozner (119) reach similar conclusions: "low plasma values do not necessarily indicate a scorbutic process unless such values exist concomitantly with a deficiency of the white cell-platelet content," and "a steady depletion or continued lowering of the plasma value and subsequently the white cell-platelet layer indicates a dietary shortage, and when the white cell-platelet layer levels become completely depleted scurvy follows."

Experience with the production of all grades of deficiency disease in human subjects will be necessary before the dietary standards can be made as satisfactory as possible. Even then the standards will, as emphasized above, be chiefly of value in assessing the average intake of groups; it is to be expected that they can be improved in accuracy so far that one may be justified in diagnosing the presence of deficiency disease in a proportion of a population whose average intake is sufficiently far below the standard. This cannot be done with the recent "optimal" standards which have been used so widely. It must be realized that the standards for some vitamins were fixed in the absence of any observations on human beings on which to base them; as Sinclair has said, ". . .our knowledge of these comes at present partly from guess-work and the guesses have not always been very inspired" (6).

There is a real need for two advances in the formulation of dietary standards. In the first place, a dual standard should be drawn up; a higher standard to serve (as the Recommended Dietary Allowances of the National Research Council was intended to serve) as a "tentative goal toward which to aim in planning practical

dietaries" (36), and a lower standard representing the closest possible approximation to the average *requirement* for the maintenance of the individual in the second zone of nutriture defined above. This lower standard would then be one with which to compare dietaries in order to assess their *adequacy*; a given dietary could not be classed as *deficient* because it was below the higher standard, but could be so classed if it fell below the lower standard. If such dual standards were available, there would be much less tendency for the uncritical to claim that deficiency diseases are rife because the dietary intake fails to reach the higher standard. The second much-needed advance is to attach to the standard for each nutrient, (which has the properties of a mean) the appropriate measure of deviation in order that it may indicate the extent to which the standard requirement varies from one individual to another, as pointed out by Pett (120).

From what has been said it is clear that the widely held belief that general dietary improvement in this country will result in greatly decreased morbidity, increased productivity, more satisfactory personal adjustments and other evidence of "more positive health" remains a hypothesis. This may not be subject to direct test, but it would appear that the investigation of possible correlations of these qualities with the level of nutriture of well chosen samples may point the way toward further studies to explain the significance of any correlations which are found to exist. Such studies might be profitably undertaken within a population which has already been under careful observation.

The present widely held views regarding human nutrition, alleging the widespread occurrence of deficiency diseases in this country, have been based on uncritical acceptance of doubtful diagnostic criteria and on the improper application of tentative dietary standards. The confusion and contradictions mentioned in the opening paragraph have arisen from a failure of some workers in this field to use their critical faculties in digesting the available observational data and in devising experiments to test their hypotheses adequately. There is an urgent need for an author who, in the words of Funk, will write "with the intention of giving a summary of the modern investigations, and by means of a careful selection of references to facilitate the research for anybody who wishes to read the original literature. This careful selection (is) absolutely necessary, for there is perhaps no other subject in medicine where so many contradictory and inexact statements (are) made, which instead of advancing the research retarded it by leading investigators in a wrong direction" (121).

Further advance can only come through a great deal of carefully controlled work on human subjects. Progress in the last 10 years has been disappointing, largely because "fed by inadequate evidence a fire of uncritical enthusiasm to detect and treat imagined deficiencies has swept our countries, its flames fanned by certain political and vested interests" (6). Progress will not be possible until the present position is viewed clearly. Humiliating though it may be, it is necessary to realize how imperfect is our scientific knowledge of human nutrition for application to the conditions of men and of nations, before we can begin to extend that knowledge in an intelligent way. Meanwhile, in applying the knowledge we already possess, "We must base our demands on our positive knowledge,

not on the gaps in our knowledge" (108, p. 89) and we must firmly reject the palpable absurdities which are offered us. For the future we require a far more comprehensive account of the biochemistry and physiology of the vitamins before we can answer satisfactorily many of the problems which physicians and public health administrators pose today. Of one thing we may be reasonably sure; the recent tendency to find the answer to most public health problems in terms of nutrition will disappear. The introduction and universal consumption of an ideal diet would not ensure abounding health; it would lay only one of the foundation stones on which such health might be based. A multitude of environmental factors, including as not the least important the individual's reaction to his fellow members in human society, must be in correct adjustment before such health can ensue (122).

SUMMARY

A classification and definition of zones of nutriture (or degrees of nutritional status) is made as follows: Saturation (with sub-zone of excess); unsaturated, but functionally unimpaired; potential deficiency disease; latent deficiency disease; clinically manifest deficiency disease. It is suggested that the second zone represents adequate or good nutriture, and that there is no cogent evidence that any higher level is more beneficial to health in the human; in this case, "optimal" nutriture can not be differentiated from adequate nutriture.

The data for assessing nutriture are of three types: those obtained by clinical examination, by biochemical and physiological tests, and by measurement and estimation of the food intake. Clinical examination detects only the well developed deficiency disease and must be supplemented by other evidence. Recent claims that the diagnosis of deficiency diseases of some of the vitamins may be made solely on the biomicroscopic examination of certain tissues have been weighed and found wanting. The interpretation of most of the biochemical tests is uncertain because these tests have not been correlated with the production and cure of the clinical signs of the disease in human subjects. The dietary standards which are widely employed in estimating dietary adequacy were not designed as standards of adequacy and are not founded on sufficient data on human subjects; consequently they give false excessive measures of the incidence of deficiency conditions. The estimates of widespread deficiency disease in this country (brought together in Bulletin 109 of the National Research Council) depend largely on estimation of the dietary intake and upon biochemical tests, and are therefore unreliable.

Future advances in the means of appraising the nutriture of the individual will rest upon elaboration of our knowledge of the biochemistry, physiology, and pathology of the vitamins in man. This can be attained 1, by investigations in which clinical deficiency diseases are produced and cured in a considerable number of subjects; 2, by careful studies of those spontaneously occurring cases of dietary deficiency disease and of conditioned deficiency disease. Such studies will lead to more accurate evaluation of the nutriture of populations, and facilitate the search for correlations between nutriture and the quality of health of a population.

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THE PROTEINS OF PLANTS

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The existence of proteins in plant tissues was first recognized by the Italian natural philosopher and physician Beccari in 1728. His observations were not published until 1745 (1), but he demonstrated in a lecture given at the earlier date that wheat flour contains a substance which yields an alkaline liquor on dry distillation and which also putrefies, when exposed to the air in a warm moist place, in a manner indistinguishable from the behavior of what he termed "animal substance." These crude tests were adequate to show that the gluten prepared by washing wheat flour dough with water until the starch is removed is entirely different from the starch itself, and to permit its classification as a substance allied in composition with the flesh of animals.

During the last part of the eighteenth century and the first two decades of the nineteenth, such investigators as Rouelle (2), Fourcroy (3), Einhof (4), and Taddei (5), together with many others, greatly extended the knowledge of this and of other analogous products obtained from plants. To Fourcroy we owe the term albumin and the observation that plant saps contain substances allied in solubility and composition to the white of egg; to Einhof the observation that a part of the gluten of wheat flour is soluble in alcohol and that similar products can be prepared from rye and barley. He also noted that leguminous seeds contain albuminous substances different from those present in wheat. Taddei separated wheat gluten into two well differentiated parts by means of alcohol and designated the alcohol-soluble component gliadin.

The fact that these plant products contain nitrogen as well as carbon, hydrogen, sulfur, and oxygen was apparently first clearly recognized by Fourcroy, but the earliest comprehensive attempts to determine their composition were made by Boussingault (6) in 1836, by Mulder (7) in 1838, and by Liebig and his students (8, 9, 10) in 1841. Mulder stated his conviction that proteins are the "foodstuff of the entire animal kingdom and are probably synthesized only by the plants," an idea that was seized upon by Liebig and developed into a broad generalization which maintained that there are only four kinds of protein in nature, albumin, casein, fibrin, and gelatin. The animal was supposed to acquire these proteins from plants either directly in the case of graminivorous animals, or indirectly in the case of carnivorous animals. The part played by the animal was merely, to quote the striking phrase used by S. W. Johnson (11, 12) in 1867, that it "moulds over these vegetable principles into the fibrine, albumin and casein of its muscle and other tissues, of its blood, milk and other secretions."

Although greatly oversimplified, these views placed extraordinary emphasis upon the importance of plant proteins and, accordingly, it is rather surprising that so little attention was paid to these substances during the second half of the nineteenth century. Many observations of great significance were of course

made in this period by one or another of the leaders in chemistry and physiology, but only three investigators of great eminence chose to devote their whole attention to the fundamental problems of the chemistry of the proteins of plants. These were Ritthausen, Schulze, and Osborne.

Ritthausen (1826–1912) is remembered today as the investigator whose careful and painstaking studies of the proteins of plant seeds, extending over the period from 1862 until his retirement from the professorship at Königsberg in 1899, laid the foundation of our knowledge of these substances. His book "Die Eiweisskörper der Getreidearten, Hülsenfrüchte und Ölsamen" (13), published in 1872, stated many fundamental principles that must still be considered by all who work with seed proteins, and for the first time suggested that the determination of amino acids is the most satisfactory chemical method for the characterization of proteins, a suggestion that lay dormant for thirty years for want of suitable analytical techniques. In addition, Ritthausen discovered both glutamic (14) and aspartic (15) acids as products of the hydrolysis of proteins, and he was also able to show that Liebig's views regarding the limited number of proteins in nature required modification inasmuch as he encountered a number of seed proteins which could not be fitted into Liebig's highly simplified scheme. Nevertheless, Ritthausen was content to group together under the same designation all proteins from whatever source that corresponded with each other in their solubility relations and had approximately the same composition with respect to carbon, hydrogen, nitrogen, and sulfur. These criteria were, in fact, the only ones available to him for the characterization of proteins in spite of his belief that the determination of the amino acids produced by hydrolysis would furnish superior analytical evidence of difference or identity as the case might be. His work was indeed carried out at the dawn of amino acid analytical chemistry and no suitable quantitative methods were developed until Kossel (16) entered the field in 1898 and devised methods for the determination of the basic amino acids.

Schulze (1840–1912) is the first of the students of plant chemistry whose interest and accomplishments in the field of metabolism entitle him to be regarded as a biochemist in the modern sense of the term. His work was begun in 1872, and he concerned himself for many years with the chemical phenomena that occur when plant seeds sprout and develop into seedlings. One of the most conspicuous of these events is the disappearance of the reserve protein of the seed and the enrichment of the tissues of the young plant in asparagine, the amide of aspartic acid. Schulze showed, by analytical chemical methods, that there was insufficient aspartic acid in the proteins of the lupine seeds with which he worked to account for the very large quantities of asparagine that were formed, and accordingly he concluded that this substance arose as the result of a secondary process. It required many years of the most painstaking research to unravel the nature of this process, but in 1898 (17) he was in a position to state that, under normal conditions, the protein of the seed is hydrolyzed by proteolytic enzymes to amino acids, that these are oxidized with the production of ammonia, that the ammonia then combines with what he called nitro-

gen-free residues, which were presumably derived from the carbohydrate metabolism, to form asparagine, and that the asparagine is transported into the growing parts of the plants where it serves as one of the most important sources of nitrogen for the synthesis of new protein in the developing tissues. This hypothesis of the metabolism of protein in seedlings has stood the test of subsequent investigation and remains, with minor elaboration, the basis of our present day views.

Schulze's direct contributions to protein chemistry are equally fundamental: he discovered phenylalanine in 1879 (18), and arginine in 1886 (19), both as products of the metabolism of proteins in lupine seedlings. Glutamine, the amide of glutamic acid, was first isolated from the water extract of beet-root tissue in 1883 (20). Schulze thus shares with Emil Fischer the unique distinction of having discovered no less than three of the amino acid components of the intact protein molecule.

Osborne (1859–1929), the third of the great nineteenth century plant protein chemists, did the greater part of his outstanding work on the isolation of the proteins of seeds during the last decade of the century. Because of his later investigations with Mendel upon the nutritive properties of proteins, which extended from 1910 until his retirement in 1928, Osborne's work is far more widely known than is that of the other two. Nevertheless it is apparent, from the more recent literature that deals with the preparation of seed proteins, that few present day students of these substances take the time to study Osborne's early papers with the attention they merit. References and credit are given, to be sure, but it is seldom that a full appreciation of what he really accomplished is indicated.

Osborne's work was done before the development of modern theories of the solubility of proteins made generalizations upon the effect of the salt concentration and hydrogen ion activity of the solvent possible, to say nothing of the effect of the dielectric constant. The concept of the isoelectric point, the use of buffer solutions, even the general acceptance of the view that proteins are ionizable electrolytes which can combine with acids and with bases, all came later, although on this last point Osborne was himself entirely clear. Yet in all of his early work, Osborne demonstrated a remarkable if instinctive appreciation of the fundamental relationship between the solubility of the protein and the salt content, the acidity, and the temperature of the solvent, and brought about his separations of the protein components of the extracts from seeds by well-conceived manipulations of these factors. For the isolation of the most soluble components, he was accustomed to dialyze the aqueous solution into alcohol, thereby altering the dielectric constant of the solvent and precipitating these substances in a form suitable for further study. In 1902, he published two papers on the basic character of the protein molecule (21) in which he showed that edestin, a typical seed globulin, enters into ionic reactions with acids to form true salts;[1] in 1905 he published a solubility curve of this same protein

[1] The first of these papers deals with the behavior in aqueous solution of a compound, that edestin forms with acid, which decomposes spontaneously to liberate the edestin in

(22) in salt solutions which showed both the ascending limb of the salting-in and the descending limb of the salting-out effects. These were pioneering attempts to formulate the principles of solubility upon which all of his protein isolation studies had been predicated.

During the early part of his work, Osborne was still more or less under the influence of the older ideas that assumed identity between proteins of different origin provided that they had the same solubility behavior and elementary composition. The advent at the turn of the century of the simple nitrogen distribution method of Hausmann (23) and of the powerful analytical methods of Kossel, enabled him to subject his preparations to more searching analysis with the result that he soon became convinced that there were few if any cases in which the same protein occurs in different plant species. He came to regard the demonstration of difference as being far more important than that of similarity and later, with the collaboration of Wells (24), was able to push these investigations to their logical conclusion by the application of immunological tests. The outcome was the generalization that, with two or three relatively unimportant exceptions in which the experiments were inconclusive, all known seed proteins are specific substances each being different in some respect from the others.

Up to the conclusion of this phase of Osborne's work, the concept plant protein had been more or less restricted to the fairly well known proteins of seeds. Schulze had given some attention to the metabolism of proteins in the growing green tissues, and agricultural chemists were, of course, concerned with the analytical determination as well as with the nutritive properties of the proteins of green fodder plants. Green leaf cells had long been recognized as being rich in proteins, but there had been few attempts to isolate these substances and very little was known of their properties. In 1920, however, Osborne and Wakeman (25, 26) in this country and Chibnall and Schryver (27) in England independently began their studies of the proteins of the green leaf which were subsequently continued for many years by Chibnall. Techniques were developed whereby samples of protein could be prepared from leaf tissues and subjected to chemical analysis. It was apparent from the first, however, that the products obtained, although reasonably pure with respect to gross contamination with carbohydrates or pigments, were far from homogeneous with respect to protein components, and they were invariably secured in a denatured condition with a solubility that differed profoundly from the native material characteristic of the living cell. Nevertheless, these preparations served admirably for the study of the nutritive properties of leaf protein and also for the investigation of the amino acid composition, although their properties could shed little light upon the chemical behavior of the protein systems of the living cells.

Chibnall's more recent investigations were thoroughly reviewed by him in his Silliman lectures delivered at Yale University in 1938 (28). In these he pointed

an insoluble condition. He says, "This derivative of edestin is formed by hydrolysis, the amount formed being proportional to time and the concentration of the solution in hydrogen ions," a statement that has a thoroughly modern ring.

out clearly that the preparations of leaf proteins with which he had been concerned were undoubtedly mixtures of many components of more or less similar solubility relations; the only discrimination possible with existent techniques was that between the protein associated with chlorophyll and other pigments and lipids in the chloroplasts and the colorless and almost lipid-free material derived from the cytoplasm of the cell.

The concept plant protein has thus been broadened in the last quarter century to include not only the seed proteins but also an entirely new class of products. These substances have now become available, although usually in an altered and denatured form, but there is no reason to assume that improvements in methods will not ultimately make it possible to secure specimens in an unaltered form for study. In fact, as will be pointed out, some advance in this direction has already been made in the case of the chloroplastic proteins.

PLANT PROTEINS. It may be well to consider in some detail just what is meant by the term plant protein. In the first place, the word protein is used with two fairly clearly distinguishable implications. The more restricted use applies it to specific substances which have frequently received designations and which, in the ideal case, have been demonstrated by chemical and physical tests to be homogeneous. Although it is doubtful that this ideal has been fully attained in more than a few, even if any, specific cases in the plant field, the statement that gliadin or zein or edestin is a protein is not ambiguous, or is at least no more so than the corresponding statement for the animal proteins such as ovalbumin or hemoglobin. The mental reservation that is made concerns the individual preparation of the substance. A given preparation probably contains a greater or less proportion of contaminating material, or may be a system of components that differ only slightly from each other. Nevertheless, however crude, it is an approach to an ideal, and it is to this ideal that the specific designation, or the general term protein belongs. We employ exactly the same restriction when we say that sucrose is a sugar. Here, the chances are that the sucrose, even if purchased at the grocery, contains only a small fraction of one per cent of contaminating material other than moisture. In the case of a protein preparation this percentage will doubtless be considerably larger.

The term protein is also employed in a more general sense as in the phrase seed protein or leaf protein. Here, the emphasis is upon the sum total of the substances in the seed or leaf which as individuals conform to the restricted definition of the word. In this sense, protein is a collective noun, and it is implied that a given preparation is a mixture of more or less related substances. The term purification as applied to material in this category refers to the removal of contaminants of non-protein nature.

The classification of proteins is an old problem to which no adequate answer has even yet been given. It is fairly easy to discriminate between, for example, the alcohol-soluble prolamins of the cereal grains and the salt solution-soluble globulins of many kinds of dicotyledonous seeds. Solubility is and will probably remain for many years the basis of classification, but the weaknesses of

this approach become more and more obvious as our knowledge broadens. An albumin is said to be soluble in water while a globulin is soluble only in the presence of salt. Yet the solubility curves of both albumins and globulins show a rising limb as the salt concentration is increased to a certain point, and a descending limb on further increase. It would be difficult indeed to draw a line on such a diagram and say that all proteins the solubility of which places them above this line shall be called albumins, all below it globulins.

The classification of plant proteins is rendered the more difficult by the fact that they occur in nature almost invariably in complex mixtures and thus, by interaction, modify each other's solubility. Accordingly as procedures for the isolation of individual components are carried out, the operator encounters what appear to be changes of the solubility. These sometimes may even lead him to entertain the suspicion that the solubility of the component in which he is interested has been altered during the experimental procedure, although this suspicion may, in fact, be groundless.

For practical purposes a rough classification of plant proteins on the grounds of location in the plants is, however, possible. This is the classification that has been tacitly used in the above discussion, and divides these substances into the proteins of the seeds, which serve primarily as the nitrogenous nutriment of the developing embryo, and the proteins of the physiologically active tissues of the leaves and stalks, and presumably also of the roots. The distinction bears many analogies to an attempt at a classification of animal proteins on the basis of whether they are derived from the hen or from the egg and has similar disadvantages. Nevertheless, just as a moderate degree of success attended early efforts to isolate and purify the chief proteins of the egg, so a considerable degree of success has been attained in the isolation and purification of many seed proteins. However, it is true that, with the conspicuous exception of animal proteins such as some of those found in milk or in blood, or in certain glands and specialized tissues, our knowledge of the individual proteins of the actively metabolizing cells either of the plant or of the animal is limited indeed.

Seed proteins. The higher flowering plants are divided botanically into two main groups according to, among other fundamental differences, the structure of the seeds. The dicotyledons produce seeds that more or less readily split into two halves, or cotyledons, located between which is the embryo which develops into the young plant when the seed is exposed to the proper conditions. The monocotyledons on the other hand, the group to which the cereal grains belong, bear seeds with only a single analogous storage organ usually called the endosperm, which is, in most of the cereals at least, characterized by the presence of a considerable amount of starch. The embryo is attached to the endosperm.

The cotyledons, or endosperm, are in all cases the locus of the essential food materials required by the embryo for its initial development, and this food is laid down chiefly in the form of the relatively stable substances, starch, protein and fat, together with a moderate proportion of inorganic material. The enzyme systems essential for the normal metabolic processes are likewise present,

and the entire structure is a living and respiring organism (29) in which life can persist often for many years provided that the seed is maintained in a dehydrated condition. When exposed to the proper conditions of moisture and temperature, the enzyme systems come into full action and the phenomena of growth are initiated.

Just as in the case of any other living organism, proteins of many different kinds are present, and it is therefore not surprising that there is scarcely a single species of plant seed the protein content of which can be accounted for at all adequately in chemical terms. The proteins of wheat have probably been studied more thoroughly and extensively than those of any other seed; yet the recent monographic treatment of the subject by C. H. Bailey (30) indicates that there is no general agreement among the large number of investigators who have concerned themselves with these proteins as to what they are or as to the chemical relations among them. Bailey concludes his chapter on the prolamins with the statement, "That various gluten protein preparations are not 'pure' in a strict chemical sense seems obvious. Whether these preparations are merely mixtures of a limited number of chemical entities, or are made up of a graduated and well-nigh infinite number of components, extending regularly in their chemical and physical properties from one extreme to the other, is not fully apparent." His discussion of the glutelins indicates that even less that is clear and definite is known about this obviously complex group of components of the protein system, whereas only fragmentary information has been recorded concerning the water-soluble and salt solution-soluble proteins, the so-called albumins and globulins of the seed.

Dicotyledonous seeds in general contain as their main protein component one or more globulins which, in the case of many of the oil seeds such as the nuts, hempseed, tobacco seed and the seeds of the cucurbits, can under correct conditions be obtained in crystalline form. Although in itself no guarantee of homogeneity (31), the separation of crystals suggests that the solid phase is at worst a relatively simple mixture of closely allied components and that there is no gross contamination with non-protein material. Most of the information in the literature of seed proteins refers to preparations of these main globulin fractions. They are assumed, and with a high degree of probability, to represent the chief storage protein of the cotyledons;[2] as a rule they appear to be relatively stable substances and can be subjected to repeated separation from solution or to recrystallization without conspicuous change in properties. Rigid tests of homogeneity have been only rarely applied to products of this type; however K. Bailey (34) has pointed out that even his most carefully prepared and purified samples of crystalline edestin, although homogeneous in the ultra-

[2] Whether or not the so-called storage proteins share to any degree in the metabolism of the resting seed is unknown. However they are intra-cellular proteins and can be recognized by suitable microscopic technique as granules which in the case of certain oil seeds are indistinctly crystalline (32). During the period when the seed is reaching maturity and again when the process of sprouting has become established, these proteins undoubtedly take an active part in the metabolism (33).

centrifuge, had a solubility that was to a large extent dependent upon the solute-solvent ratio either in polar or non-polar solvents. Fractions which differed from each other in gross solubility behavior could be obtained, but no fraction obeyed the ideal solubility laws. Accordingly even in this especially favorable and well-known case, chemical homogeneity cannot be claimed.

It is unfortunate that the powerful electrophoretic methods that have been so strikingly successful with many kinds of animal proteins are not in general applicable to the seed globulins. These substances are so insoluble at the low temperature and ionic strength conventionally used for such experiments that successful records of the moving boundaries cannot be obtained unless one is prepared to work at strongly alkaline reactions. Fontaine[3] has, however, secured evidence that three main electrophoretic components are present in the globulin fraction of the peanut. Two of these could be provisionally identified with the arachin and conarachin of Johns and Jones (35) but, inasmuch as the analysis was carried out at a pH somewhat greater than 9, the interpretation of the results is still tentative.

In addition to the main storage globulin or globulins which can usually be readily isolated, dicotyledonous seeds invariably contain other protein components. The literature provides little information regarding the relative proportions in which any of these substances are present. Analytical methods whereby different individual proteins can be distinguished are poorly developed and about all that is usually attempted is to record the relative proportions of the total nitrogen of the seed that are soluble in a series of solvents chosen so as to bring successively into solution the main classes of proteins that are expected to be present. Preparations that are presumed to represent these main groups are then isolated and subjected to more detailed analysis, but it is obvious that, with the possible exception of the major storage protein, there is little or no assurance that the fractions represent other than complex mixtures.

As a rule, detailed amino acid analyses have been obtained only for the chief globulin fraction, and it has been the custom, for want of more complete information, to attempt to assess the nutritive effect of the total protein of a seed from what is known of this component. The fallacy of this treatment of the data may be illustrated by the case to which attention has recently been called by Jones (36). Arachin, the main globulin of the peanut, has been found to be deficient from the nutritive point of view in both tryptophane and methionine but conarachin, a globulin present in smaller proportion in this seed (8 per cent of the fat-free meal as against 25 per cent of arachin) (37), yields relatively high proportions of these two amino acids (38, 39). Nutritive supplementation therefore occurs when the whole protein of this seed is fed, whereas a deficiency would be apparent if the experiment were restricted to the main globulin. In many, if not most other cases, knowledge is indeed limited to the amino acid composition of the main globulin and, to make the matter worse, few even of

[3] Personal communication from Dr. T. D. Fontaine of the Southern Regional Research Laboratory, New Orleans. The analysis was made by Dr. R. C. Warner of the Eastern Regional Research Laboratory, Philadelphia.

these proteins have been studied save with respect to the more easily determined amino acids. As a result, the efforts to provide analytical information upon which to base a judgment of the nutritive value of the whole protein of such an important food material as the soy bean have been gravely circumscribed in spite of the urgent necessity for such information that has arisen from the present war conditions. This whole problem has been discussed in detail in a recent symposium (40).

It may not be out of place to draw attention to the nature of the mixture of proteins that an extract of a seed may be expected to contain. Aside from the main storage protein or proteins, usually globulins in the case of the dicotyledonous seeds, there should be present components that represent the proteins of the embryo and also the enzymes of both cotyledon and embryo tissue. Furthermore, there is no reason to assume that the whole of the cotyledon protein is storage protein; there are presumably residues of cytoplasmic and nuclear protein, derived from the cells of the cotyledons, some of which may be brought into solution during the process of extraction, and in addition the proteins derived from the tissue of the seed coat must be considered although these doubtless form a relatively small part since the seed coat seems to consist mostly of substances allied to the carbohydrates (41), and to contain little nitrogen.

In general, it is impossible to bring into solution all of the nitrogen of a seed when solvents appropriate for proteins alone are used. As an example, the experience of Smith and Circle (42) with fat-extracted soy bean meal may be quoted. These observers have studied the proportions of the seed nitrogen that are dissolved or "dispersed" over a wide range of conditions. In the absence of added salt, the curve for the soluble nitrogen passed through a rounded maximum at about 85 per cent in the vicinity of pH 2, dropped rapidly to a rounded minimum at a level of about 10 per cent at pH 4, which is in the isoelectric range of the main protein components, and rose rapidly between pH 5 and 7 where it flattened out at about 85 per cent, this quantity increasing gradually to 95 per cent at pH 11 to 12. In the presence of added salt, a family of curves was obtained depending on the concentration of the sodium chloride employed. At low salt content (0.01N), the curve differed but little from that with water; with 0.1 N salt the maxima were a little lower and the minima a little higher; with 0.5 N salt the maximum at pH 2.5 was at 70 per cent, the minimum at pH 4.2 was at 42 per cent, and the curve then rose rapidly and followed a nearly straight line course from 73 per cent at pH 6 to 92 per cent at pH 11. The curves as a whole provide the most complete picture available of the basic solubility relations of the proteins of this seed and somewhat similar data have recently been published for the proteins of the peanut by Fontaine and his associates (43). It should be emphasized perhaps that the determinations were made with the use of entirely empirical and conventional method of extraction, but the observations are nevertheless strictly comparable *inter se* and doubtless represent a fairly general phenomenon.

Of the 10 to 12 per cent of the soy bean nitrogen that passed into solution in water at pH 4, about half was not rendered insoluble by reagents that are

ordinarily used to precipitate protein. This quantity therefore furnishes an approximate measure of the non-protein soluble nitrogen of the seed, such substances as asparagine, choline, and adenine together with amino acids and simple peptides being presumably represented. Furthermore, that part of the nitrogen in this fraction that was precipitated by the reagents tried may be regarded as a rough measure of the proportion of the true water-soluble proteins of the seed, that is, of the proteins that conform to the usual definition of the term albumin. The measure is indeed rough since the solution analyzed contained that part of the isoelectric protein that was soluble in the volume of solvent and at the temperature and salt concentration employed, while no allowance is made for the albumin-like proteins that may have been present in the precipitate either adsorbed upon insoluble protein or in combination with it.

The part of the seed protein that resisted extraction with saline solvents at alkaline reactions presents a difficult problem. In the first place, the quantity observed would be expected to be a function of the success of the grinding operations to which the meal had been subjected. Only if there were a presumption that nearly all of the cells had been ruptured and that time had been allowed for adequate diffusion of the contents could one assume that this fraction represents definite insoluble components. However, Smith, Circle and Brother (44) have furnished data that indicate that their grinding operations were effective, and there seems little reason to doubt that much of the insoluble residue represents protein different in nature from the soluble material. The quantity is perhaps too great to make reasonable the assumption that it represents the protein components of the embryo although these might well be in part included since they are presumably allied in behavior to the proteins of leaves; such proteins are usually difficult to extract with the customary protein solvents after the tissue has been dried.

It is superficial to dismiss this insoluble part of the seed protein as representing a portion of the main protein that had become denatured during the operations of grinding and extraction. Such products are usually soluble at slightly alkaline reaction, whereas 5 per cent or more of the total seed nitrogen remained insoluble even when a solvent as alkaline as pH 11 was used.

The main protein components of the soy bean were obtained from the extract by Smith and his co-workers by isoelectric precipitation at about pH 4.1. They describe conditions under which they recovered approximately 84 per cent of the seed nitrogen in the form of a dry product suitable for many industrial applications, and presumably also satisfactory for the investigation of the nutritive properties of this part at least of the total protein. Nevertheless, that material of this kind is probably far from homogeneous from the point of view of the protein chemist was made apparent by some recent unpublished experiments carried out in this laboratory. The object of these was to secure by modern methods, from a known variety of soy beans, specimens of protein that should represent as nearly as might be the globulin glycinin described many years ago by Osborne and Campbell (45). Accordingly soy beans of the variety

Illini[4] were ground and extracted with petroleum ether, whereby most of the fat content was removed, and, after the solvent had evaporated, were further ground until all passed through a fine sieve. The meal contained 7.00 per cent of protein nitrogen and 0.37 per cent of non-protein nitrogen. About 91 per cent of the protein nitrogen could be brought into solution when the material was thoroughly extracted in a Waring Blendor with 6 per cent sodium chloride solution adjusted to pH 7.1 to 7.5 by the addition of barium hydroxide. The suspension was subsequently centrifuged in a cup centrifuge and the residue washed. The solution, or "dispersion," so obtained was extremely turbid and remained so even after passage through a Sharples supercentrifuge. This last procedure threw down, in the form of a sludge in the centrifuge bowl, a product high in ash as well as nitrogen, and the somewhat clarified solution contained only about 75 per cent of the seed protein nitrogen. Owing to the impossibility of obtaining clear solutions either by centrifugation or filtration, after extraction of the meal by this technique, experiments were next carried out in which the meal was repeatedly ground in suspension in either 6 or in 10 per cent sodium chloride solution adjusted to pH 7.1 to 7.5. The suspension was thickened by the addition of sufficient filter paper clippings to make a soft coherent mass, was again passed through the mill and was then formed into cakes wrapped in drilling and pressed between steel plates in the hydraulic press. The solvent of lower salt concentration still gave a turbid solution after the extract had been passed through the supercentrifuge, but that of 10 per cent concentration yielded a solution that was markedly superior in appearance, being almost clear. This method of extraction is one that has repeatedly been found to be effective with many kinds of seeds but, in the present case, the clarified extract again contained only about 75 per cent of the protein nitrogen. There was little sludge deposited in the Sharples centrifuge bowl and it was obvious that the apparently more effective treatment with the Waring Blendor, with respect to the proportion of the protein nitrogen that was extracted, merely represented the dispersion of a protein component, or more accurately fraction, that was so far from being in true solution as to be easily sedimented in the supercentrifuge. Its physical properties were different from those of the main globulin which was present in true solution, but there seems little doubt that a similar material would be present in preparations of the type discussed by Smith and his associates.

Inasmuch as complete clarification of the solution is an essential prerequisite to the preparation of a protein fraction free from non-protein contaminants, attention was next turned to the use of even more concentrated saline solutions for the extraction. It was found that saturated sodium chloride solution, adjusted in reaction after being mixed with the meal as before, gave extracts that were perfectly clear both by transmitted and reflected light after being passed

[4] The beans were obtained through the courtesy of Prof. W. C. Rose of the University of Illinois; they were designated "Illini beans certified M13." The experiments were carried out by Dr. G. W. Pucher and Mr. L. S. Nolan of this laboratory.

slowly through the supercentrifuge. They deposited only a trace of sludge in the bowl. Obviously this solvent failed to bring into suspension or solution those components of the meal to which the unusual turbidity of ordinary extracts is due. On the other hand, it extracted only about 60 per cent of the total protein of the seed, a result not unlike that of Smith and Circle who found that saturated sodium chloride solution extracted only 73.6 per cent of the total nitrogen of Illini soy beans under the conditions of their experiments. Nevertheless, extracts prepared in this way proved to be admirable for the isolation of the main globulin fraction. After dialysis, and adjustment of the reaction of the supernatant solution to pH 5.0 by the addition of sufficient acetate buffer of pH 4.7 and ionic strength 1.0,[5] the protein separated in a form that could be readily centrifuged in the cup centrifuge, and could be purified by being suspended in a liberal volume of 10 per cent sodium chloride solution and redissolved at pH 8.0 by the addition of 0.2 N sodium hydroxide. This solution was then clarified and again dialyzed, and the protein was flocculated at pH 5.0. After being dehydrated with acetone and dried with exposure to the air, the preparations contained between 16.9 and 17.0 per cent of nitrogen and 0.2 per cent of ash, calculated moisture free. Such preparations represent as nearly as can be ascertained the "glycinin" described by Osborne and Campbell, but the product derived from Illini soy beans differed in nitrogen content from the one described by those workers. Their glycinin contained 17.53 per cent of nitrogen corrected for ash and moisture although its solubility relationships were closely similar. Evidence given by Csonka and Jones (46) suggests that the main globulin fractions obtained from soy beans of different varieties differ from each other in nitrogen content, a point which is in itself indicative of lack of homogeneity, and this may be the explanation of the failure of the present experiments to duplicate Osborne's preparation. The material was, however, found to be identical in histidine content with a preparation made many years ago by the salt precipitation method under Osborne's personal direction (47).

The yield of globulin was in most cases close to 20 per cent of the weight of the fat-extracted meal. Since this meal contained 7.0 per cent of protein nitrogen, it may be assumed that about 40 per cent of protein was present in it; thus the preparations of purified main globulin obtained represent only about half of the total protein of the seed. Although low, this yield is higher than that recorded by Osborne and Campbell who obtained only 16 per cent of the meal as globulin that could be separated by dialysis.

Obviously the total protein of soy beans represents a complex mixture of components. It was found possible to secure preparations in a yield as high as 30 per cent of the meal by treatment of a clarified and dialyzed extract with diluted alcohol to a final concentration of 40 per cent at pH 5.0, but the product was low in nitrogen and high in ash content. The product undoubtedly represented a mixture of many components since there were present not only the main globulins but also the more soluble globulins that separate incompletely when

[5] The buffer solution is made by mixing 250 ml. of 4.0 M sodium acetate with 75 ml. of glacial acetic acid and diluting to 1000 ml.

the dilute dialyzed solution is adjusted to the isoelectric range as well as the proteins originally soluble in water alone. The preparations contained much denatured and insoluble material and their behavior shed little light upon the general nature of the proteins of this seed. Nevertheless, suitable modification of this technique may lead to effective fractionation.

Legume seed proteins are far less satisfactory to deal with in the laboratory than are the proteins of the oil seeds, and the experiments just described furnish an illustration of this fact. Yet it was from a legume seed, the jack bean, that Sumner was able to isolate crystalline urease (48), and his achievement in obtaining at least three other protein components in crystalline form (49) remains as a demonstration that the isolation of what are presumably the individual proteins of legume seeds is not a hopeless undertaking, difficult though it may be.

This section may be concluded with some remarks on the directions that it would seem desirable for future research on seed proteins to take. Two quite different motives make such research essential. In the first place, those who are interested in nutrition, especially in the vitally important problem of the selection of sources of plant proteins to serve as substitutes for the more highly esteemed and widely used proteins of animal origin, require comprehensive and trustworthy data on the amino acid composition of the whole protein of many kinds of plant seeds. Much of the available data refer, as has already been mentioned, only to specimens of the more easily prepared proteins and these may represent as little as one-half of the total protein that is made available on ingestion of the seed meal or of protein-rich concentrates from it. Techniques must therefore be developed whereby samples can be obtained that represent the whole protein, or at least a large part of it, in a form so purified from carbohydrate and other contaminants that the fractional losses of amino acids that occur during hydrolysis will not significantly affect the analytical results. Whether one or more than one fraction is secured is immaterial so long as the quantitative relation to the seed meal is ascertained and the fundamental data established that will permit the calculation of the amino acid composition of the entire seed. The opinion is widely held that the hydrolysis of seed meals themselves, or of grossly impure protein concentrates from them, is a feasible approach to the solution of this problem. Control analyses are occasionally presented to show that hydrolysis of a protein in the presence of starch or of a sugar results in small if not wholly negligible losses of certain amino acids (50). Unfortunately such controls are not entirely convincing. They assume that no other kind of component is present in the material hydrolyzed which can interfere by giving rise to humin at the expense of the amino acids. Yet Chibnall (28, p. 140) and Lugg (51) have both strongly emphasized the danger that arises from the presence of contaminants of plant origin in samples of proteins subjected to hydrolysis. The special contaminant with which they were concerned is almost invariably found in preparations of proteins from the leaf, and they carefully point out that its chemical nature is still unknown. However, the evidence suggests that it may be allied with the pentosans or pectins or possibly with the substances classified as mucilages. At all events, experi-

ments carried out in this laboratory (52) have shown that the humin nitrogen produced during the hydrolysis of edestin may be increased from a mere trace to as much as 9.7 per cent of the total nitrogen if furfural is added, or to 5 per cent in the presence of pectin, this last quantity being increased still further if cellulose were also added. The loss of 5 per cent or more of the protein nitrogen in the humin cannot but have serious effects upon the apparent amino acid composition, especially if it is remembered that the losses probably fall unevenly upon the various amino acids present.

The other motive for further research upon seed proteins is admittedly more academic, but it would seem that the time is now rapidly approaching when the whole problem of the nature of the seed proteins should be reinvestigated with the aid of the modern physico-chemical techniques that have made possible the striking advances of recent years in such a field as that of the blood proteins. The system of protein components in an extract prepared from a seed meal is quite different in properties, especially in solubility, from such a system as that in blood, or milk, or an extract from a gland, but there is no reason to assume that its details cannot be worked out equally as successfully, although doubtless the methods will have to be modified to meet the special needs of the case. The inapplicability to the seed globulins, for example, of the present procedures for the measurement of mobility in the electrophoresis apparatus is indeed a challenge to those concerned with the development of this extremely useful device. The application of modern theories of protein solubility to the problem of the separation of these proteins from each other would appear to offer no insuperable fundamental difficulties.

Research in the past, both with animal and with plant proteins, has been too often restricted to the investigation of a single or at most a small group of components of a given tissue. Where this component has special physiological activities or interests, this is justifiable, and in those cases where the component has favorable physical and chemical properties, gratifying success has often attended the efforts. But the time has come when a broad and far more detailed view of the chemical composition of living organisms is becoming essential to our understanding of their behavior, and it is well to remember that the simple plant seed, the ultimate source of all foodstuffs, is one of the most important organisms with which mankind is concerned.

Leaf proteins. Knowledge of the normal protein components of plant tissues other than the seeds is in a far less satisfactory state than is that of the seed proteins. It is only recently that a moderate degree of understanding has been obtained of the relationship between the chlorophyll and the chloroplastic proteins in leaves, and that trustworthy information on the composition of the proteins of the leaf cytoplasm has become available, but there is little that can be considered even yet as systematic information about these substances. With respect to the proteins of other tissues there is direct evidence from chemical studies that proteins are to be found for example in bark (53), or in fruits (54), or in rubber latex (55), and some of these products have been subjected to more or less conventional investigation for amino acids although little has been ascertained about their solubility relationships in the native state.

It is an interesting commentary upon the effectiveness of modern physico-chemical methods of research on proteins that the only leaf proteins concerning which a truly comprehensive body of knowledge should have been accumulated in recent years are the virus proteins, substances that are formed in leaves as a result of specific diseases and which are themselves the agents whereby these diseases are transmitted. In this field alone has the full armamentarium of modern physico-chemical research been employed by skillful and enthusiastic workers and the results have indeed been striking. The reasons for this are not far to seek; the virus proteins of diseased leaves were promptly shown to be type substances characteristic of virus disease in general and their investigation was accordingly greatly stimulated by the medical implications of this fact. Their enormous molecular weight and convenient solubility made it possible to isolate them by physical methods, and their physiological activity even in most extreme dilutions rendered tests for their presence a fairly simple matter so that their behavior could be far more precisely followed than is the case for almost any other kind of protein. Thus we are today in the anomalous and logically indefensible position of knowing a great deal more about the proteins of leaves that are suffering from a serious disease than we do about those of normal and healthy leaves. Knowledge of animal disease, especially that of man, rests securely upon a foundation of knowledge of normal anatomy, physiology, and biochemistry: although botanists have provided adequate anatomical information with respect to normal plants, far too little has been learned of their physiology and biochemistry.

Examination of a leaf under the microscope shows it to consist of cellular tissue with clearly defined walls. The cells are filled with a more or less jelly-like colorless material called cytoplasm, which contains what are termed inclusions of several different kinds. Most important from the biological point of view is the nucleus, the site of the chromosomal material, but the most conspicuous are the chloroplasts. These are tiny green structures present in enormous numbers and in them the pigments of the leaf are concentrated. In addition, particularly in older cells, a central region filled with a clear liquid of low viscosity, and termed the vacuole, is to be distinguished. These different anatomical structures imply the presence of different chemical structures and chemical tests indicate that proteins are present in each of them. One might expect, therefore, that chemical examination of leaves would reveal several kinds of proteins of which two would be found in large proportions. These would represent the protein associated with the green pigment, the chloroplastic protein, and the other would be free from pigment and would represent that of the cytoplasm. In addition, if techniques were devised to separate the very small proportion of the nuclear material, one would anticipate that it would be allied in behavior and composition with the nuclear material from the better known animal sources, and would be found to be rich in nucleic acid. Lastly, if the vacuole fluid could be separated, one might expect to find that it contains a little protein in solution, although from the low viscosity this could not account for any large part of the total leaf protein.

Chloroplastic protein. That the chlorophyll of the green leaf is present in

some sort of combination with substances of high molecular weight has long been suspected. Willstätter (56) in 1922 pointed out that the solubility behavior of the pigment in the leaf suggests that it is not free in molecular solution but is in large part in what he held to be an adsorbed condition. Lubimenko (57) at about the same time was willing to go further and postulated a probable chemical combination between the pigment and the protein component of the chloroplast. Noack (58) in 1927 provided strongly suggestive evidence of this. He developed a technique for grinding leaves with sand, centrifuging off the debris, and then subjecting the turbid green fluid to high-speed centrifugation whereby a sediment was obtained that appeared to consist chiefly of the chloroplasts themselves although in a shrunken condition. He noted that the characteristic red fluorescence of the chlorophyll in this sediment was extinguished when a sample was heated for a few seconds to a temperature that exceeded 70° although it returned if the heating were prolonged. Both protein and the fluorescing chlorophyll were precipitated by the addition of ammonium sulfate or of lead acetate to a suspension of the sediment, but the fluorescence was again extinguished if the precipitate were heated, and treatment of the sediment with proteolytic enzymes likewise led to extinction. He interpreted these experiments to mean that the chlorophyll is normally combined with or adsorbed upon the protein in such a manner that denaturation or digestion liberates it whereupon it passes, in the aqueous medium, into colloidal aggregates that no longer show detectable fluorescence. The return of the fluorescence after more prolonged heating he considered to be due to solution of the chlorophyll in waxy components of the tissue, a view later supported by the experiments of Smith (59).

These observations were followed up ten years later by Menke (60) in Germany and by Granick (61) in this country. Menke employed a differential centrifugation technique with 0.66 M primary potassium phosphate as fluid phase, and, by discarding the top and the bottom layers of the sediments, secured material 99 per cent of which he thought to consist of the chloroplasts derived from the spinach leaves used. Analyses showed that the solids of this product contained 47.7 per cent protein (N × 6.25), 37.4 per cent lipid, 7.8 per cent ash, and 7.1 per cent undetermined material. The lipid fraction, obtained by extraction with ether followed by ether-alcohol, was extremely complex in composition. In addition to chlorophyll-a and -b, carotin and xanthophyll, fatty acids, glycerides, phosphatides, sterols, and wax-like components were said to be present.[6]

Preparations that represented concentrates of the protein components of the chloroplasts, and termed "chloroplast substance" by Menke, were obtained, among other ways, by treatment of the turbid suspension with one-quarter of its volume of saturated ammonium sulfate solution. The flocculent green precipitate that separated was found to contain 56.4 per cent of protein (N ×

[6] Chibnall and his associates have carried out careful analytical studies upon leaf lipids. Their data, which agree with these statements of Menke completely, are summarized in Appendix III of his published Silliman lectures (28, p. 284).

6.25), 31.9 per cent of lipid, 4.7 per cent of ash, and 7.1 per cent of undetermined material. After being freed from lipids, the residue consisted chiefly of presumably denatured protein of a corrected nitrogen content of 14.6 per cent which was insoluble in water, dilute acid, alkali, or salt solutions, but could be dissolved in part in 50 to 60 per cent alcohol in the presence of a little alkali. Extraction with very dilute aqueous alkali removed a small proportion of a phosphorus-containing protein component which apparently contained nucleic acid. The residual material was still, however, contaminated with carbohydrate, and its behavior was reminiscent of that of certain preparations obtained in 1920 from alfalfa leaves by Osborne and Wakeman (25) and in 1926 by Chibnall and Grover (62). Subsequently Menke (63, 64) provided analytical information upon the content of chlorophyll as well as upon the inorganic constituents of these preparations.

Granick (61, 65) introduced what seemed to be an improvement in the method of preparing chloroplasts by grinding the leaves in the presence of hypertonic sugar solutions. With the use of 0.5 M glucose or sucrose, he was able to secure sediments of chloroplasts in which the proteins were apparently not denatured and which remained normal in appearance for several hours after isolation. Tests showed them to be able to produce oxygen in the presence of light for several minutes. Determination of the quantity of chlorophyll a + b in the isolated sediment and in the whole leaf permitted an estimate of the relative proportion of the total chloroplasts of the tissue that had been isolated, and from this ratio and the nitrogen content of the sediment it was possible to obtain a reasonable measure of the relative proportion of chloroplast nitrogen in the leaves. For the tobacco and tomato leaves that he studied, he thus found that from 30 to 40 per cent of the *total nitrogen* of the leaf is present in the chloroplasts. Of the nitrogen of the chloroplasts, 13.2 per cent was present in the ether-alcohol extract of the lipids, 6.7 per cent was soluble in trichloroacetic acid solution, and the remaining 80.1 per cent therefore presumably represented protein. The lipid nitrogen consisted of chlorophyll nitrogen to the extent of about 10 per cent of the total chloroplast nitrogen and the balance of 3.2 per cent was assumed to represent such components as phosphatides. It was further calculated that from 35 to 45 per cent of the *protein nitrogen* of the leaf was present as the protein of the chloroplasts, thus leaving from 55 to 65 per cent for the other proteins, chiefly those of the cytoplasm. These estimates, while admittedly approximations, furnish the orders of magnitude of the quantities concerned and have been substantially confirmed by Neish (66) who worked with several other species and extended the observations to a consideration of the inorganic components. This worker, however, employed water extracts, or rather suspensions, and noted that the chloroplasts themselves swell and disintegrate in water with granulation of their contents. The preparations he studied consisted of this granular material which was flocculated by the addition to the suspension of calcium or magnesium salts in a concentration of 0.1 M. They therefore more closely resembled the "chloroplast substance" of Menke than the presumably intact chloroplasts studied by Granick.

Hanson (67) in Australia has also confirmed the observations of Granick, in particular the point that the chloroplast proteins make up about 36 per cent of the total protein of the leaves of a grass species. In addition he concerned himself with the weight ratio between the protein and the chlorophyll in the chloroplast. He found this to vary from 4.5 to 5.4 under various metabolic conditions.

Smith (59) has applied refined spectroscopic techniques to the study of the properties of extracts prepared from spinach and aspidistra leaves by grinding the tissue with slightly alkaline buffer solutions. Thus, although he occasionally speaks of the results as applying to the chloroplasts, his preparations were doubtless suspensions of the granules derived from the disintegrated chloroplasts and were more nearly like those of Menke and of Neish. He confirmed early observations such as those of Hagenbach (68) that the absorption band in the red shown by chlorophyll as present in the leaf is shifted slightly towards the blue if the pigment is extracted from the leaf with organic solvents. He also noted a similar shift when his extracts were treated with reagents, or by procedures, that bring about denaturation of the protein. The observations with detergents were especially striking. If digitonin were added to the turbid leaf extract, this at once became clear and showed the characteristic shifts in the spectrum. The behavior of the protein in the solution was likewise greatly modified. It could no longer be precipitated even by saturation of the solution with ammonium sulfate, but after prolonged dialysis, whereby most of the detergent was removed, could be precipitated by the addition of one-tenth of the ammonium sulfate necessary for saturation of the solution. This last property distinguishes such material sharply from that of the protein in the original untreated extract. Careful chemical analyses of products purified by repeated salt precipitation or by differential centrifugation showed that the protein nitrogen content was 7.4 per cent, the protein content thus being (N $\times$ 6.25) 46.5 per cent, remarkably close to that of similar preparations of Menke. The chlorophyll content was 7.86 per cent and thus 100 parts of protein were associated with approximately 16 parts of chlorophyll. This figure differs slightly from the ratio found by Hanson, but this worker employed chloroplasts, isolated by the Granick procedure, which might well contain a surplus of protein other than that combined with pigment.

Smith has also studied the effect of other detergents than digitonin, especially that of sodium dodecyl sulfate (69) which was found to convert the chlorophyll in the protein complex into phaeophytin by removal of the magnesium at a rate dependent on the hydrogen ion concentration. At constant pH, the rate was proportional to the concentration of the detergent. Evidence that the protein component is denatured by the treatment was obtained from its solubility behavior. The combination between pigment and protein was not broken, however, in spite of this change, and it was accordingly concluded that magnesium plays no part in the linkage between protein and green pigment.

Considerable light was shed upon the relationship between the protein and the pigment in the presence of detergents by the study of Smith and Pickels

(70) of the behavior of the solutions in the ultracentrifuge. Untreated extracts of the chloroplast material were found to sediment at relatively low speeds (2500 to 3000 r.p.m.) with a purely random spread of particle size. Thus the original extracts are in fact suspensions of particles that are not in true solution. The addition of detergents to these suspensions converted them into brilliantly clear solutions, but the effects of the several reagents were different. The apparatus permitted the observation of sedimenting boundaries both by light absorption, thus showing the behavior of the pigment or pigment-protein complex itself, and by a refractive index gradient system that revealed the behavior of the dissolved protein in the gravitational field. Furthermore, the light absorption measurements could be made both with red and with violet light filters thus permitting discrimination between the behavior of the carotinoid pigments and the chlorophylls.

When digitonin, sodium desoxycholate, or bile salts were added to the extracts, evidence was found for the decomposition of the protein-chlorophyll complex. The situation with the first of these substances was complicated by the fact that digitonin itself is present in micellar form (71). There was a light absorption boundary which indicated that these micelles combined with or adsorbed the chlorophyll. However, a second boundary was observed from the refractive index gradient picture which was not present in the absorption picture, and this sedimented at a rate corresponding to a sedimentation constant of 13.5 S. With sodium desoxycholate and bile salts, there was no evidence whatever by light absorption for the sedimentation of the pigments, although the refractive index system showed the presence of a boundary which sedimented at the same rate as that just mentioned. Accordingly it was clear that all three detergents dissociate the pigment from the protein, which then sediments separately, the constant being the same in all three cases.[7]

The effects of sodium dodecylsulfate were widely different from these. The removal of magnesium from the chlorophyll has already been mentioned, a reaction that is rapid in the presence of acid being complete in a few minutes at pH 4.8 although requiring several hours at pH 7.5, and a day at pH 8.9. At any of these reactions, however, the pigment sedimented together with the protein, the boundary observed by light absorption being identical with that observed by refractive index increment. Furthermore, the light absorption boundaries seen in the two regions of the spectrum were likewise identical indicating that both the chlorophyll and the carotinoids were attached to the protein, either to particles of the same molecular size and shape or to the same particles.

The rate of sedimentation was much slower than that of the protein component observed in the presence of the other detergents. The sedimentation constant was 2.56 S when the protein concentration was low and the detergent concentration was 0.25 per cent. At higher protein concentration (about 1.3 per cent) and a ten times higher concentration of detergent, the sedimentation

[7] An incidental observation was made that, in concentrated urea solution, the pigment is likewise dissociated from the protein.

constant was 1.69 S indicating still further dissociation of the protein molecules although the pigment remained attached to the protein.

In the absence of measurements of the diffusion constant, calculations of the molecular weight are impossible; nevertheless Smith and Pickels offered the estimate that the protein of sedimentation constant 13.5 S found in the extracts that had been treated with digitonin or bile salts would have as a minimum a molecular weight of 265,000. The particles present when sodium dodecylsulfate is added are smaller although apparently reasonably homogeneous in size distribution, those observed in a 2.5 per cent solution of detergent being about half the weight of those in 0.25 per cent detergent.

To summarize the chemical information that has been obtained from these recent investigations of chloroplasts and their contents, it should first be mentioned that the chloroplasts are present in the cells of leaves and other green tissues in relatively enormous numbers. Estimates have been made that run to the order of several hundred thousands per square millimeter of leaf surface. An excellent review of the available information concerning them has been given by Weier (72). From many points of view, the chloroplasts bear an analogy to the red cells of blood. Since they contain the chlorophyll of the leaf, they are the locus of the reactions by means of which carbon dioxide is reduced, starch is formed, and oxygen liberated. Accordingly they are structures provided with all of the enzyme systems necessary for these activities. They are apparently bounded by a semi-permeable membrane and, when freed from the cellular tissue in the presence of water or dilute salt solutions, swell and break open thereby liberating their contents in the form of granules that readily pass into colloidal suspensions. On the other hand, if the leaf cells are ground in the presence of hypertonic solutions of sugars, the chloroplasts can be secured in a not too seriously damaged condition and they preserve some at least of their functions for a short time. It is obvious therefore that chloroplasts are available for chemical study and, although it is unfortunately much more difficult to secure preparations of them than it is of the red cells of blood, there is reason to believe that many of the chemical and physiological methods that have been employed in the study of red cells are equally applicable to chloroplasts.

The observations that have been made indicate that the chemical system within these structures is very complex. Considered in the gross, the chloroplasts contain about 35 per cent of the total protein of the leaves from which they are prepared, all of the chlorophyll and probably the greater part of the other pigments and much of the lipids. They are high in inorganic constituents (64, 66), and Menke's analyses suggest the presence of carbohydrates as well. Certainly starch is present when their photosynthetic functions have been exercised immediately before isolation.

Exactly how complex the protein system of the chloroplast may be does not appear from the evidence at hand. Menke observed a small proportion of what seemed to be a nucleoprotein component and one would anticipate the

presence of a certain proportion of enzyme proteins.[8] The bulk of the protein, however, appears to consist of components that are perhaps to be classified as lipo-proteins; the evidence now seems complete that the chlorophyll is combined with this protein as a so-called prosthetic group and the same may be true of the carotinoids. It is possible that both types of pigment are combined with the same protein molecule.

Attempts have been made (63, 67) to deduce stoichiometric ratios between the protein and the chlorophyll but these have been predicated on wholly unsubstantiated assumptions. Such ratios will have significance to the chemist only when preparations of the lipo-protein have been made that meet the criteria for a pure homogeneous substance. The molecular size of the chlorophyll protein is still unknown although Smith and Pickels' estimate gives an order of magnitude. At all events, the protein is several times larger than hemoglobin and is very much smaller than the virus proteins of leaves studied by Stanley and his associates.

Future investigation of this type of leaf protein will doubtless be immeasurably assisted when a clearer understanding of the nature of lipo-proteins in general has been obtained. It is to be hoped that techniques now available and in process of development for the study of the lipo-proteins of blood serum may find an application in this field.

Cytoplasmic protein. The isolation of the proteins of the cytoplasmic substance of the living leaf cell presents a chemical problem of the greatest difficulty. Here at least one can be relatively certain that he is dealing with a complex mixture for the cytoplasm is the seat of the chemical reactions that are associated with life itself. It is accordingly a dynamic system and there is every reason to suppose that the proteins are among the most important and perhaps the most reactive of the substances which share in the processes that take place. Present day views of the significant part played by the proteins in the chemical reactions of the animal body have been formulated for the most part as a result of the fundamental researches of Schoenheimer and his associates with isotopic nitrogen. Far from being a stable structure which remains for a long time unchanged, the individual cell protein molecule is today recognized as a unit which in all save the most inert tissues has a remarkably short life. That a similar situation probably exists in the plant cell is a logical assumption, and evidence to this effect was obtained in 1940 by Vickery, Pucher, Schoenheimer and Rittenberg (73) who observed that isotopic nitrogen is taken up by the proteins of the tissues of both tobacco and buckwheat plants in excess of the quantities that could reasonably be accounted for by growth alone during the experimental period. Similar results have been reported by Hevesy, Linderstrøm-Lang, Keston and Olsen (74) in the case of the sunflower plant. Furthermore the unique reactivity of glutamic and aspartic acids in the nitrogen exchange reactions of animal tissues was also found to characterize their behavior in the

[8] Neish has demonstrated catalase and carbonic anhydrase functions in material prepared from disintegrated chloroplasts.

plant. This last point is not, however, a matter for surprise because of the unique position that the amides asparagine and glutamine have long been recognized to hold in the metabolism of plant tissues.

Accordingly, although one can doubtless learn to isolate preparations that represent protein components of the cytoplasm, there is no reason whatever to expect such preparations to be homogeneous from the chemical point of view. Quite the contrary is to be anticipated. Although chemical examination may give information on the over-all average composition, it will reveal little concerning the individual substances present, and because of the instability of these proteins it is improbable that the preparations secured by any of the methods hitherto suggested will resemble the original material of the cell at all closely in its physical and chemical properties.

The isolation of preparations that represent a part at least of the protein of leaf cell cytoplasm in a form uncontaminated by proteins derived from the chloroplasts was probably first accomplished by Chibnall in 1923 (75). It had long been known that the vapors of chloroform or ether, or aqueous solutions of these and similar substances, would so affect the permeability of plant cell membranes as to permit the vacuolar fluid contents to exude. The reaction is irreversible, if allowed to proceed for more than a few minutes, and results in the death of the cell. In his previous work, Chibnall (27) had made frequent use of ether water as a plasmolyzing reagent in order to facilitate extraction of proteins from leaf cells, but in 1923 he noted that when leaves were immersed directly in ether, they at once lost their turgidity, and that mere pressure with the fingers would suffice to express a large part of the fluid contents. This observation revealed the possibility of treating whole leaves in such a way as to remove the vacuole fluid without rupture of the cell walls.

Chibnall found that, after treatment of the leaves in bulk with ether, it was possible to squeeze out most of the vacuole fluid with the hydraulic press, and to wash away the remainder by alternately allowing the tissue to imbibe water and pressing it out several times. The object of this operation, from the chemical point of view, was to remove water-soluble components before attempting to bring the proteins into solution. The vacuole fluid so obtained contains very little protein in solution but may contain 25 per cent of the total leaf nitrogen. Furthermore a substantial proportion of the inorganic constituents which might later contribute to the ash content of the protein preparation could thus be removed. After this treatment, the residual tissue mass was left with its cells for the most part unbroken and still containing the jelly-like cytoplasm with its inclusions of chloroplasts and other formed elements. Moreover, it was in a physical condition that made the grinding operation for the rupturing of the cell walls relatively easy. After being first ground in a meat grinder and then repeatedly passed in dilute suspension in water through a plate-type mill to afford maximum shearing action on the cell walls, the tissue residue was removed with the aid of a silk screen and a green turbid suspension that contained much of the protein of the leaves was secured. This was filtered through a thick pad of paper pulp whereby the green pigment and all of the turbidity were re-

moved. It was at this point that Chibnall accomplished the fundamental separation of the two main types of protein components of the leaf. The chloroplastic material including both chlorophyll, other lipids, and protein, being present under these conditions as a suspension of particles, as has already been pointed out in connection with the recent work of Smith and Pickels, was removed by the efficient pulp filter while the filtrate contained that part of the cytoplasmic protein that had passed into true solution. A qualification is perhaps necessary here; the filtrates obtained were clear, with only a small Tyndall effect and gave every appearance of being true solutions but, until such extracts have been studied by modern physical methods, it cannot be asserted that they are, in fact, true solutions of cytoplasmic protein. Assuming this for the present, however, the next step was the flocculation of the protein by the addition of the requisite amount of dilute acid; in general, such proteins are least soluble in the range pH 4 to 5. The precipitated protein was removed and was usually further purified by being dissolved in the least possible amount of dilute alkali and reprecipitated with acid. It was finally collected and washed and dehydrated with graded strengths of alcohol. In successful preparations, the first alcohol washes should contain at most traces of green color; if more were present the filtration step for the removal of the chloroplastic material must have been inefficient. Final extraction with ether as a rule sufficed to remove a residual trace (1 to 2 per cent) of lipid.

Chibnall has applied this method to the examination of a wide variety of leaf proteins, especial attention being given in more recent years to the grasses because of their agricultural importance. Some of the preparations were practically free from non-protein contaminants as evidenced by their high nitrogen content. A preparation from spinach leaves (76) contained 16.25 per cent of nitrogen and one from *Vicia faba* leaves (62) 16.77 per cent. Most of them, however, were in the range from 14 to 16 per cent and doubtless contained non-protein material although the nature of this and whether it should be regarded as a contaminant or as an intrinsic part of this kind of protein is still to be determined (see 28, p. 138 and p. 278 for a discussion of this point).

A vitally important feature of these experiments, and one that has scarcely been commented upon save by Chibnall himself, is the question of yields. Even in the especially favorable case of the spinach leaves, only about 14 per cent of the leaf nitrogen (19.4 per cent of the leaf protein) was isolated in the form of the cytoplasmic protein and from *Phaseolus multiflorus* leaves only 16 per cent was obtained. In most other cases where successful preparations were obtained, the yields ranged from 1 to 8 per cent, while a number of species were encountered with which the method failed entirely to yield cytoplasmic protein. In the case of the grass proteins to which much attention was given (77), a return was ultimately made to the use of ether water rather than of ether itself as the plasmolyzing agent and many successful experiments were then carried out. In these circumstances too, however, the yields were often disappointingly small and were always erratic; it became clear that some highly important factor was being overlooked or at any rate was not being controlled.

Before going on to discuss the later experiments which threw some light on the situation, it may be well to stop and consider the chemical conditions in the leaves that are being subjected to this process. In the first place, the effect of the ether or ether water upon the semi-permeability of the cell is a phenomenon of both biological and chemical import. When cells treated in this way are examined under the microscope, the entire protoplast is seen to have shrunken away from the cell wall and to be located at one end of the cell. The fluid of the vacuole has mostly passed through it, and the cell is filled with what is presumably a mixture of vacuole fluid and absorbed ether water, if this reagent has been used. The delicate equilibria upon which the life of the cell depends are obviously completely upset, and the phenomena that occur subsequently are clearly in the province of the chemist rather than in that of the biologist. By the successive pressing and washing operations that are next carried out, the vacuole fluid is washed away and the tissue is then thoroughly ground in the presence of a considerable volume of water. During this operation, the proteins of both chloroplasts and cytoplasm pass into what Chibnall speaks of as colloidal solution. What happens to the chloroplasts would seem to be reasonably clear from the results of Menke and others that have already been discussed. It can be assumed that they are disintegrated and that their contents are liberated in the form of the granules that Menke terms chloroplast substance and which Smith and Pickels found were in fact gross particles that had a wide distribution with respect to size. At all events, their practically complete removal by the subsequent efficient filtration operation would be expected. The crux of the question is what happens to the jelly-like mass of the cytoplasm exclusive of the chloroplasts. If this is indeed a thixotropic gel, it is possible that it may be dispersed by the mechanical effect of the grinding and, owing to the dilution with water, may then be converted into a form that will pass through the filter; more or less of it may pass into true solution. Obviously, until such solutions have been examined in the ultracentrifuge and in the electrophoresis apparatus we are scarcely in a position to do more than guess. However, certain points that may have their influence on the outcome of any individual preparation are more or less obvious. In the first place, a part of the gel at this stage may consist of protein that has become denatured as a result of mechanical action during grinding or of the action of surface forces. Chibnall places some emphasis on this possibility and, until we know much more of the physical and chemical properties of proteins of this kind, we shall be unable to assess its validity. But possibly more important is the fact that the proteins of the gel are now exposed to the action of a solvent of very low ionic strength at a reaction that is the result of chance. No buffer save the protein itself is present, and experience shows that these solutions are usually only a short distance removed from the isoelectric range of the proteins since only small quantities of acid are required to flocculate them. It would seem therefore that the conditions that have been reached are those more apt to promote instability of the protein systems involved than stability, and perhaps it is not surprising that success with this method of preparation is sometimes erratic. Experiments to test this view of the situation are clearly required.

However this may be, Chibnall and his associates (78) in 1933 described a technique by means of which some, at least, of the difficulties of the earlier method have been overcome. As a result of a chance observation, they found that ether water that had been used once for the plasmolysis of a batch of leaves was thereafter far more efficient for use in the treatment of subsequent batches. It was noted that the effect of such ether water upon the protoplast was somewhat slower and that it did far less violence to the cells inasmuch as marked shrinkage of the protoplast did not take place. Nevertheless, the permeability of the cell was destroyed as effectively as before and the removal of the vacuole fluid was equally easy. After the tissue was ground, the suspension of the proteins was worked up as usual, but the yield of protein after flocculation of the filtered solution was greatly increased. In a series of some seventeen species, mostly grasses, the yields ranged from 7 to 31 per cent of the leaf protein, with an average of about 18 per cent, and the experiments on repetition were far more satisfactorily consistent than with the earlier method.

Chibnall later pointed out (28) that the chemical conditions, if "used" ether water is employed, are indeed widely different and that the effect of the whole preparation method is changed. Whereas, in the earlier method the clear filtered solution of the cytoplasmic protein showed only a moderate Tyndall effect, the solutions now obtained exhibited this property to a marked degree. Furthermore, appreciable quantities of lipids were present in the preparations and, most important of all, far less protein remained on the filter in combination with the lipids; most of it passed into solution and was found in the filtrate. In other words, the specimens of leaf protein secured by this revision of the technique consisted not only of the soluble cytoplasmic protein but also contained a substantial part of the chloroplastic protein that had been combined in the tissue with the lipids. It appeared possible that the effect of the new reagent was in some way to dissociate the bond between lipid and protein and permit chloroplastic protein to pass into solution.

Chibnall has discussed this still unexplained effect in terms of the wide differences that are to be observed under the microscope between leaf cells that have been treated on the one hand with ether water and on the other with "used" ether water (28, p. 152 ff.). He points out that the new reagent is taken up by the leaves during the process in far larger amounts than is freshly prepared ether water, and offers as a speculation that the reagent, in contact with the outer surface of the protoplast, may in some way peptize the lipid which is acting as a protective colloid to the protein gel. The gel is thereby rendered available to solvents after the cells have been opened by the grinding operation. The effect was shown in the 1933 paper to be due not to a lowered concentration of the ether but to substances which diffused from the leaf cells, that is to say, to admixture with a small proportion of the vacuole fluid.

The new technique made possible the isolation of protein preparations that represented a substantially larger part of the total protein of the leaf cells, and for many purposes, such as the assay of the amino acid composition of these proteins, were a great improvement over the earlier preparations. Analysis of such samples of the whole protein and of the cytoplasmic protein prepared by

the earlier method permitted approximate calculation of the composition of the chloroplastic protein by difference. Nevertheless the observations as they stand offer a challenge to those interested in leaf cell proteins as substances, and it is clear that the new method, inasmuch as it leads to the isolation of even more complex mixtures than before, requires further modification to permit fractionation of the components.

A purely speculative suggestion may be offered for what it is worth in the effort to throw some light on the behavior of the proteins during treatment of the cells by the "used" ether water method. The reagent used for the plasmolysis is in effect a dilute solution of the vacuole fluid to which ether has been added (28, p. 155). Chibnall and his associates (78) describe in detail an experiment in which they made up the volume of the ether water used for a series of batches of leaves by the addition of some of the juice from the press, the ether concentration being maintained by suitable additions from time to time. The significant fact here may be that the subsequent extraction after the tissues had been ground was made not in the presence of a solvent of almost zero ionic strength, as is the case when fresh ether water alone is used, but in the presence of an admittedly small but definite concentration of salt owing to less effective washing away of the fluid contents of the cells. Under these circumstances, one of the chief theoretical objections to the original ether or ether water methods is diminished in force. The extraction is made in the presence of a buffer solution of low ionic strength and the protein systems may be imagined to be thereby rendered somewhat more stable. In addition, such a solution may well be a somewhat more efficient solvent for the cytoplasmic protein.

This speculation leaves out of account the question whether or not that part of the chloroplastic protein that is brought into solution in the filtrate is dissociated from the lipid. The facts appear to be that substantial quantities of lipid do pass through the filter, and Chibnall speaks of the strikingly enhanced Tyndall effect in these filtrates. Moreover, extraction of the flocculated preparations with alcohol and ether is necessary subsequently to remove this lipid. But whether or not it is still in combination with the protein does not seem to be established. Again, the application of modern physico-chemical methods would seem to offer a hope that the situation may be ultimately clarified.

Chibnall's experiments have yielded a great deal of information upon the composition of an array of protein preparations secured from the leaves of many important agricultural species. These data bear directly upon the fundamental question of the nutritive effect of leaf proteins. He pointed out that the amino acid composition of all of these preparations was remarkably uniform from species to species and that the composition was such that good nutritive properties were to be anticipated. Among other points, for example, the long and hotly debated question of the source of the cystine in the fleece of sheep restricted to a diet of herbage was finally settled by the observation that the cystine content of the leaf proteins of such herbage is in fact adequate to account for the cystine in the fleece even of notably heavy wool-producing animals (79).

On the other hand, his experiments as a whole were designed to provide only

the preliminary data required by the protein chemist and the agriculturist. He says: "But much remains to be learned, for the existing methods often fail to extract proteins in reasonable degree of purity from leaves of many important groups of plants. Moreover, the leaf proteins, in contrast to the better-known reserve proteins of seeds, are an integral part of a complex and carefully controlled mechanism, the protoplasm; consequently, if the plant physiologist is ever to be in a position to emulate Lawrence J. Henderson's brilliant researches on blood and attempt to explain protoplasmic behavior by considering the protoplast as a physico-chemical system—an ideal to which he has not yet attained—he must know more about the conditions under which the proteins exist *in the living cells*" (28, p. 120).

Vacuole protein. Chibnall's ether method provides a technique for the separation of the fluid of the vacuole from the cells without extracting any of the components of the cytoplasm and thus makes possible the chemical investigation of this vitally important part of the system both for proteins and for simpler substances. The technique is immeasurably superior to the crude grinding and alcohol precipitation methods employed to prepare the extracts from alfalfa leaves that were examined for simpler nitrogenous substances by the writer (80) many years ago, and it is extraordinary that so little attention has been paid to the opportunity presented. Most investigators in this field have been content to work with direct alcohol or hot water extracts of the cellular tissues on the assumption that the soluble components are equally well isolated in this way, although no cogent evidence that this is so has been presented.

Chibnall himself, being interested chiefly in the proteins of the leaf, frequently heated the vacuole extract to coagulate such protein as it contained in solution. In this way he established the fact that only a small part of the total leaf protein is ever to be found in the vacuole fluid secured after plasmolysis with ether and with most species none whatever could be demonstrated (28, p. 136). For example, in the spinach leaf (76) only 0.74 per cent of the leaf solids and 1.7 per cent of the leaf nitrogen were found in the heat coagulum and the preparation itself contained only 14 per cent of nitrogen corrected for ash and moisture. A few conventional amino acid analyses of such material are to be found in his earlier papers but, aside from this, there is little in the literature that sheds light upon chemical nature or composition of this part of the protein system.

CONCLUSION

The present discussion of the proteins of plants has been presented entirely from the point of view of a protein chemist interested in proteins as chemical substances. Emphasis has therefore been placed upon the problem of their differentiation and purification in the hope that, when sufficient has been learned of them, adequate chemical and physical characterizations can be made. Only when we have learned about the properties of these components of the systems that carry out the functions of the living cell shall we be in a position to learn what these functions really are. Nevertheless, the writer is keenly aware that this treatment of the subject is entirely one-sided and distorted: for it is

the *systems* of components that carry out the functions and thus the protein chemist is only one of the team of workers, both chemical and biological, who must combine their best efforts in order that we may ultimately understand what is going on in the living cell. At present it is necessary to treat plant proteins in groups. The next phase of investigation may reveal how they can be treated as individuals, that is, as specific substances. Ultimately we may hope to learn something of the complexes or systems of inter-reacting substances the behavior of which we recognize as the life process.

The purposes of this review will have been accomplished if better definition has been given to the concept plant protein, and if a little light has been shed upon the position of our present knowledge of these substances. Nothing has been said of their metabolism. Protein synthesis in plants has recently been fully reviewed by the late Professor Petrie (81) of the University of Adelaide, the composition of leaf proteins by Lugg (82), and the virus proteins by Stanley (83), by Bawden (84), and by Hoagland (85). For the most comprehensive discussion of the more general aspects of protein metabolism in plants, reference should be made to Chibnall's Silliman lectures (28). Careful study of all of these papers will show, however, that present knowledge is and will remain largely empirical until the proteins themselves are better understood.

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